Instituto Teófilo Hernando de I+D del Medicamento
Departamento de Farmacología y Terapéutica
Facultad de Medicina
Universidad Autónoma de Madrid

HOMEOSTASIA DEL CALCIO Y SU PAPEL EN LA REGULACIÓN DE LA EXOCITOSIS EN LA CÉLULA CROMAFÍN DE LA RATA HIPERTENSA

Memoria de Tesis para optar al grado de Doctor presentada por

Ricardo de Pascual del Castillo

Directores

Antonio García García
Luis Gandía Juan

Instituto Teófilo Hernando de I+D del Medicamento
Departamento de Farmacología y Terapéutica
Facultad de Medicina
Universidad Autónoma de Madrid

D. ANTONIO GARCÍA GARCIA, Catedrático del Departamento de Farmacología y Terapéutica de la Facultad de Medicina de la Universidad Autónoma de Madrid,

y

D. LUIS GANDÍA JUAN, Profesor Titular del Departamento de Farmacología y Terapéutica de la Facultad de Medicina de la Universidad Autónoma de Madrid, por medio de la presente

CERTIFICAN QUE:

D. RICARDO DE PASCUAL DEL CASTILLO ha realizado bajo su dirección el presente trabajo titulado **"Homeostasia del calcio y su papel en la regulación de la exocitosis en la célula cromafín de la rata hipertensa"**, como Tesis para alcanzar el grado de Doctor por la Universidad Autónoma de Madrid

Y para que conste a los efectos oportunos, firman la presente en Madrid a 10 de octubre de 2013

Fdo. Antonio García García
Catedrático
D.N.I. 74.257.173-V

Fdo. Luis Gandía Juan
Profesor Titular
DNI. 22.121.980-M

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MARCO DE LA TESIS

MARCO EN EL QUE SE HA DESARROLLADO ESTA TESIS

En el Instituto Teófilo Hernando de I+D del Medicamento (ITH) y en el Departamento de Farmacología y Terapéutica (DFT) de la Facultad de Medicina de la Universidad Autónoma de Madrid (UAM) se desarrollan fundamentalmente dos líneas de investigación, una vinculada al sistema nervioso y la otra al aparato cardiovascular. La relacionada con el sistema nervioso se enfoca hacia estudios de neurotransmisión sináptica y comunicación neuronal y estudios de neurotoxicidad y neuroprotección.

Los estudios de comunicación neuronal se centran en el esclarecimiento de los mecanismos que regulan la dinámica del catión calcio (Ca^{2+}) y la liberación exocitótica de neurotransmisores. Muchos de estos estudios se han abordado desde una óptica básica-molecular y neurofarmacológica, en las últimas tres décadas del siglo XX. Sin embargo, en la última década se ha hecho un esfuerzo traslacional, impulsado por el desarrollo del Servicio de Farmacología Clínica del Hospital Universitario de La Princesa (HUP), vinculado a la UAM. Este servicio forma parte del ITH y también del HUP, y ha sido el motor del cambio de enfoque de la investigación realizada en el ITH, que apunta más directamente a su proyección a enfermedades concretas.

En el terreno de las neurociencias y la neuropsicofarmacología cabe destacar nuestro interés en la búsqueda de fármacos neuroprotectores con potencial terapéutico en las enfermedades neurodegenerativas, el ictus y el traumatismo de la médula espinal. En el terreno cardiovascular cabe resaltar nuestro interés por la hipertensión arterial y el papel que desempeña el eje simpático-suprarrenal en su desarrollo y mantenimiento.

La tesis doctoral que presento para optar al título de doctor por la UAM se relaciona, precisamente, con esta última temática, utilizando el modelo de la rata espontáneamente hipertensa para explorar la hipótesis y objetivos de esta tesis doctoral.

La tesis se ha escrito con el formato de manuscritos y artículos publicados. Incluye una Introducción, Materiales y Métodos y los Resultados con los artículos en cuestión. Finalmente he elaborado una discusión general sobre los cuatro artículos publicados, para explicar lo que he aportado de novedoso en la comprensión de los mecanismos que regulan las señales de Ca^{2+} en células cromafines de la médula adrenal, en ratas normotensas e hipertensas y su influencia sobre la secreción de catecolaminas.

Los artículos que constituyen el núcleo de esta Tesis Doctoral son los siguientes:

1. FERREIRA, R.M., **DE PASCUAL, R.**, DE DIEGO, A.M.G., CARICATI-NETO, A., GANDIA, L., JURKIEWICZ, A. and GARCIA, A.G. Quantal catecholamine release from adrenal chromaffin cells of control and hypertensive rats. J. Pharmacol. Exp. Ther. 324: 685–693, 2008.

2. FERREIRA, R.M., **DE PASCUAL, R.**, CARICATI-NETO, A., GANDIA, L., JURKIEWICZ, A. and GARCIA, A.G. Role of the endoplasmic reticulum and mitocondria on quantal catecholamine release from chromaffin cells of control and hypertensive rats. J. Pharmacol. Exp. Ther. 329: 231–240, 2009.

3. FERREIRA, R.M., **DE PASCUAL, R.**, SMAILI., S.S., CARICATI-NETO, A., GANDÍA, L., GARCÍA, A.G and JURKIEWICZ, A. Greater cytosolic and mitocondrial calcium transients in adrenal medullary slices of hypertensive, compared with normotensive rats. Eur. J. Pharmacol. 636: 126–136, 2010.

4. **DE PASCUAL, R.**, FERREIRA, R.M., GALVAO, K.M., LAMEU, C., ULRICH, H. SMAILI, S.S., JURKIEWICZ, A., GARCÍA, A.G. and GANDÍA, L. Lower density of L-type and higher density of P/Q-type of calcium channels in chromaffin cells of hypertensive, compared with normotensive rats. Eur. J. Pharmacol. 706: 25-35, 2013.

INTRODUCCIÓN

1.-INTRODUCCIÓN

1.1.-LA HIPERTENSIÓN ARTERIAL

Según define la Organización Mundial de Salud (OMS), la hipertensión arterial (HTA) es la elevación crónica de la Presión Arterial Sistólica (PAS), de la Presión Arterial Diastólica (PAD) o de ambas a la vez por encima de unos valores considerados normales. La dificultad estriba en fijar esos valores normales o en determinar cuál es la línea divisoria entre los valores considerados normales y los anormales. De este modo podríamos aceptar otra definición: "La hipertensión arterial es el nivel de PA por encima del cual los beneficios derivados de la intervención sanitaria superan a los riesgos", ahora bien en términos de parámetros objetivos podríamos definirla como: "Se puede considerar una PA elevada cuando los valores de la PAS sean superiores a 140 mmHg o la PAD supere los 90 mm Hg" (Tabla 1). De estos datos depende su prevalencia como veremos a continuación.

Categoría	PA sistólica (mm Hg)	PA diastólica (mm Hg)
óptima	<120	<80
normal	120-129	80-84
normal elevada	130-139	85-89
HTA grado 1 (leve)	140-159	90-99
HTA grado 2 (moderada)	160-179	100-109
HTA grado 3 (grave)	≥180	≥110
HTA sistólica aislada	≥140	<90

Tabla 1.-Valoración de las cifras de presión arterial según la European Society of Hypertension–European Society of Cardiology "guidelines for the management of arterial hypertension". (Mancia y col., 2007).

La prevalencia de la HTA en una población, o lo que es lo mismo, la proporción de individuos que son hipertensos en un momento dado y en una población determinada, varía en función de distintos factores como podrían ser la edad de la población en cuestión, la metodología utilizada en la medida de la presión arterial, el

número de veces que se debe determinar la presión del individuo y, sobre todo, los parámetros elegidos que delimitan la normotensión de la hipertensión.

Si se eligieran los valores clásicos de 160/95 mmHg, o el más actual de estos parámetros 140/90 mmHg, los datos de la prevalencia cambiarían drásticamente. Así, al utilizar los límites de 160/95 mmHg a escala internacional, la prevalencia de la HTA registrada oscilaría entre un 10 y un 20% de la población adulta (18 años y más). Por el contrario, si los limites utilizados son los de 140/90 mmHg, la prevalencia aumentaría hasta el 30% de la población adulta (entre 18 y 65 años). Con estos últimos valores podríamos decir que la HTA es una de las enfermedades cardiovasculares con mayor prevalencia en los países desarrollados y no tan desarrollados.

Aunque es una enfermedad en sí misma que provoca un porcentaje no desdeñable de muertes directas, la HTA actúa como un importante **factor de riesgo** de padecer enfermedades cardiovasculares, hasta el punto de que la OMS (OMS, 2012) estima que más del 60 % de las enfermedades cerebro-vasculares y casi la mitad de la enfermedad isquémica cardiaca puede atribuirse a ella. En España, la situación no es diferente a la del resto del mundo, y la enfermedad isquémica cardiaca, principalmente en los hombres, y la enfermedad cerebro-vascular, sobre todo en las mujeres, producen una elevada mortalidad, además de graves incapacidades en personas que las sufren pero no fallecen, causando así un importante problema de salud pública.

Debido a que su mortalidad directa no es tan elevada como la causada por otras enfermedades, y a que está muy extendida, la HTA es una enfermedad con la que una gran parte de la población convive sin darle la importancia que realmente tiene. La prevalencia de HTA en España se estima en torno a un 35 %, aunque llega al 40 % en edades medias y al 68 % en mayores de 65 años, afectando a más de 10 millones de personas (OMS, 2012).

Ante la magnitud del problema, es necesario tomar medidas de forma inmediata para tratar de reducir la prevalencia de la HTA, medidas que deben incluir todos los niveles de prevención y posibilidades terapéuticas y sociales. Tanto las instituciones internacionales como los gobiernos de muchos países están basando parte de sus acciones e intervenciones para la prevención primaria de enfermedades crónicas, que incluye medidas basadas en la práctica regular de actividad física y en una dieta

equilibrada y saludable, como medios de prevención del sobrepeso y la obesidad, que están asociados a la hipertensión.

1.1.1.-Fisiopatología de la presión arterial

La presión arterial sistémica está determinada directamente por el gasto cardiaco y las resistencias vasculares periféricas. El gasto cardíaco va a depender del volumen sistólico (que varía según la contractilidad cardíaca y el retorno venoso) y de la frecuencia cardiaca. Por su parte, las resistencias periféricas están reguladas por diversos factores nerviosos, humorales y locales.

Adicionalmente, sobre estos determinantes directos de la presión arterial intervienen una serie de determinantes indirectos como son la actividad nerviosa central y periférica autonómica, la reserva corporal de sodio y líquido extracelular, el sistema renina-angiotensina-aldosterona (SRAA) y hormonas locales como las prostaglandinas, cininas, factor natriurético atrial (ANP) y otros péptidos. Se sabe también que el endotelio tiene una importante participación en la regulación de la vasoconstricción y la vasodilatación arterial. Muchos de estos factores están interrelacionados en circuitos de autorregulación que consiguen mantener la PA en unos límites estrechos.

Entre estos mecanismos reguladores, es de destacar al **sistema nervioso autónomo**, especialmente el sistema simpático, que juega un papel importante en el control circulatorio por mecanismo reflejo o actuando sobre el tono vascular. Como mecanismo reflejo, responde a la estimulación de los barorreceptores aórticos y carotídeos con la liberación de noradrenalina (NA) desde las terminaciones nerviosas simpáticas, produciendo una vasoconstricción y un aumento de la frecuencia cardíaca, participando así en el ajuste rápido de la PA. Así, por ejemplo, si disminuye el retorno venoso (y en consecuencia el gasto cardíaco) o baja la PA (por ejemplo tras un cambio postural), se activa este reflejo simpático produciendo aumento de la frecuencia cardíaca y del volumen cardíaco recuperándose así la PA. En situaciones normales, este reflejo neural sirve para aumentar la PA cuando baja y reducirla cuando sube (Guyton y Hall, 2006b).

Por otro lado, los mecanismos que regulan la PA a largo plazo están principalmente relacionados con la regulación de sodio y líquido extracelular. En este sentido, el **riñón** juega un papel importante en la regulación de la PA y del balance hidroelectrolítico, reaccionando a los cambios de PA con un aumento de las resistencias

vasculares renales, excreción de sodio y liberación de renina con la consiguiente activación del SRAA.

La alteración de uno (o más) de estos múltiples factores que influyen y/o regulan el gasto cardíaco o las resistencias vasculares periféricas va a ser suficiente para iniciar un aumento de la PA que se perpetuará después como HTA mantenida. Debemos destacar que, a pesar de los notables esfuerzos de investigación realizados en este campo, en la mayoría de pacientes con HTA (más del 90%) no se encuentra una causa concreta de la misma, hablándose así de HTA esencial.

La **HTA esencial** se ha relacionado principalmente con un incremento de la resistencia vascular periférica de pequeñas arterias y arteriolas. Entre los diferentes factores que pueden conducir a este incremento de las resistencias periféricas encontramos las situaciones de **estrés.** Así, se sabe que el estrés incrementa el tono simpático, produciéndose un aumento de la liberación de adrenalina por la médula suprarrenal y de noradrenalina por las neuronas adrenérgicas activadas por estimulación del sistema nervioso central (SNC), lo cual provoca un aumento del gasto y frecuencia cardíaca por sus efectos agonistas α-adrenérgicos inmediatos. Secundariamente, el incremento de los niveles circulantes de adrenalina van a actuar sobre receptores adrenérgicos de tipo $\beta 2$ de la membrana neuronal presináptica lo que estimula una nueva oleada de liberación de noradrenalina de las vesículas de almacenamiento y origina una vasoconstricción considerablemente más prolongada. Así, los elementos sugeridos por *Folkow*, con ingredientes tales como: aumento del estrés, niveles plasmáticos elevados de catecolaminas e incremento de la actividad presora ante el estrés, están presentes en el hipertenso esencial (Folkow, 1982).

1.1.2.-Enfoque terapéutico actual de la HTA esencial

En el tratamiento de la HTA el objetivo primario es reducir el riesgo cardiovascular que esta enfermedad supone a largo plazo para el paciente, lo que requiere no solo el tratamiento de la HTA, sino también la prevención de otros riesgos modificables, entre otros el sedentarismo, el consumo excesivo de alcohol, el tabaquismo, la obesidad o la diabetes.

En la actualidad disponemos de distintas clases de fármacos antihipertensivos que pueden considerarse de primera línea y que pretenden contrarrestar las posibles

alteraciones en esos condicionantes directos e indirectos de los incrementos de la PA, entre los que encontramos los antagonistas del calcio, los bloqueantes β, los IECA, los antagonistas del receptor AT_1 de la angiotensina II y los diuréticos (Figura 1).

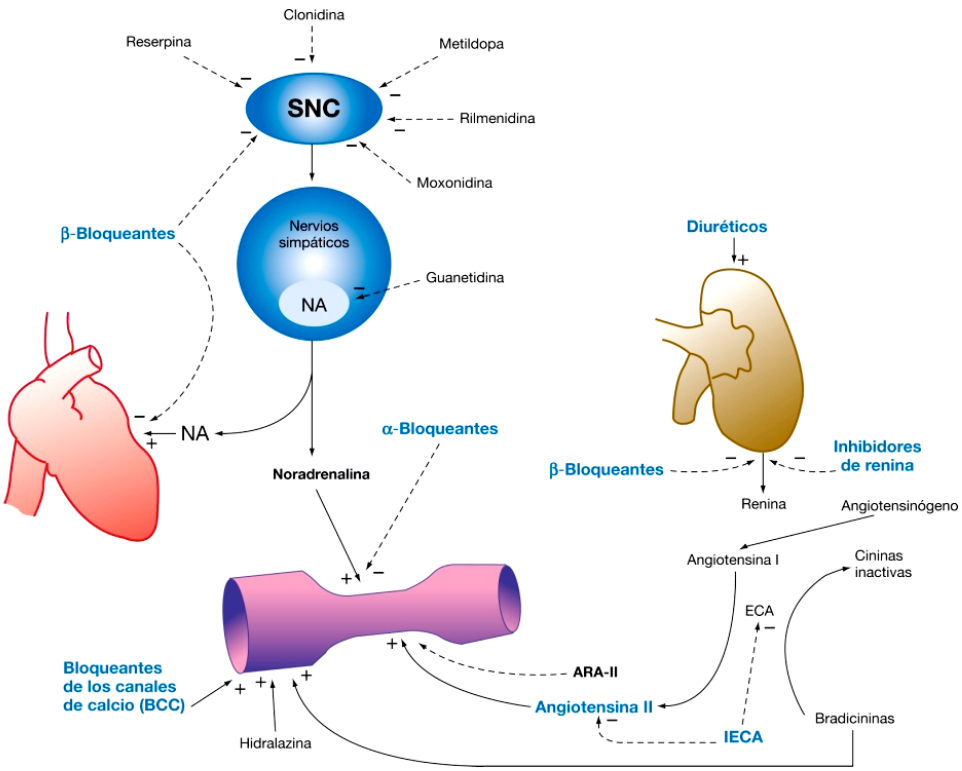

Figura 1. Fármacos de primera línea para el tratamiento de la HTA y sus órganos dianas. Tomado de *Sánchez-García, Sáiz y Flórez. En "Farmacología Humana".(Sanchez-García, 2008)*

Aunque clásicamente había gran preocupación por cuál era el fármaco antihipertensivo de elección en cada situación clínica concreta, las evidencias demuestran que la mayoría de los pacientes hipertensos va a necesitar al menos 2 fármacos antihipertensivos para lograr los objetivos de control de PA. Esto se debe a que la HTA es una enfermedad sistémica multifactorial, en la que están implicadas varias vías neuroendocrinas. En consecuencia, la inhibición de sólo 1 de ellas resulta habitualmente insuficiente, mientras que la combinación de antihipertensivos con mecanismos de acción diferentes ha demostrado una mayor eficacia en el tratamiento del paciente hipertenso.

En consecuencia, para la mayoría de los sujetos con HTA no se trata de decidir cuál es el mejor antihipertensivo, sino cuál es la mejor combinación en cada paciente.

Las ventajas de la terapia combinada incluyen una mayor eficacia antihipertensiva como consecuencia de la combinación de diferentes mecanismos antihipertensivos, a veces aditivos y otras sinérgicos, así como una menor incidencia de efectos adversos, en unas ocasiones porque son necesarias menos dosis de cada fármaco en monoterapia y, en otras, porque se ponen en marcha mecanismos compensadores. Además, las combinaciones fijas frente a las libres mejoran el cumplimiento terapéutico, lo que podría facilitar la consecución de objetivos a largo plazo.

En la Figura 2 se muestran las distintas combinaciones recomendadas en las Guías de la ESH/ESC 2007 (Mancia y col., 2007). Existen otros fármacos antihipertensivos, como los antagonistas α_1-adrenérgicos, los agonistas α_2-adrenérgicos o los de receptores imidazolínicos (moxonidina), los inhibidores de la renina (aliskireno) y los antagonistas del receptor de la aldosterona que no se consideran de primera elección, aunque pueden usarse en asociación con los fármacos de primera elección.

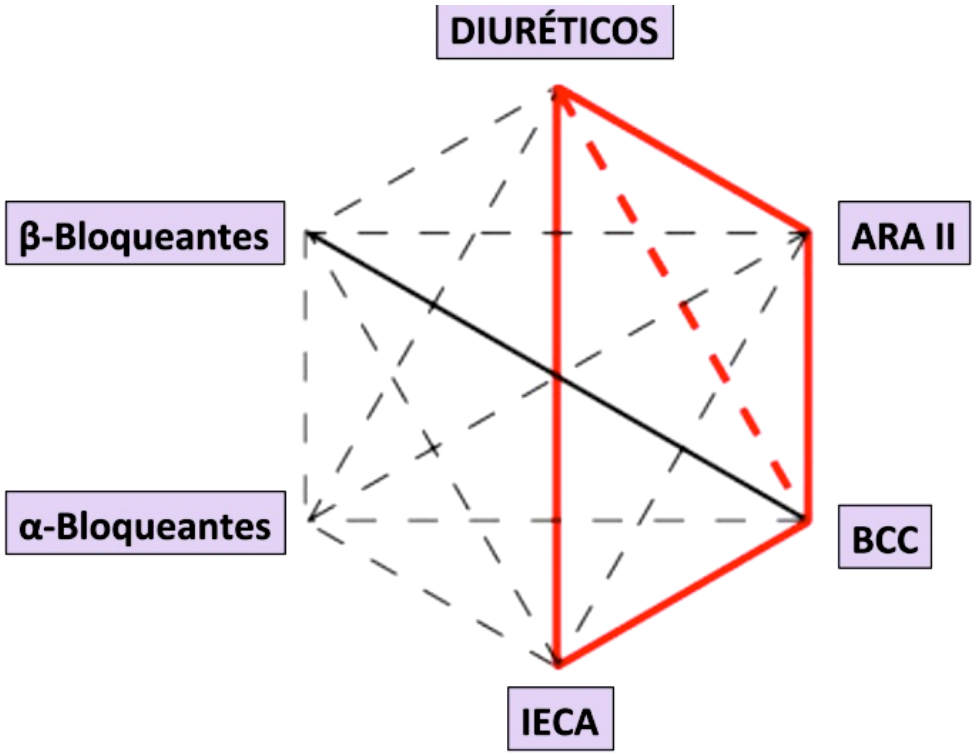

Figura 2. Posibles combinaciones de antihipertensivos (Tocci y col., 2009).

1.1.3.-Catecolaminas, estrés y presión arterial

Como he comentado anteriormente, entre los diferentes factores que pueden producir un incremento de las resistencias periféricas encontramos las situaciones de **estrés.** Se sabe que ante una situación de miedo, alarma, estrés u otros conflictos emocionales, se produce una descarga masiva de catecolaminas, tanto desde terminaciones del sistema nervioso simpático como desde la **médula adrenal**. Esto prepara de forma instantánea al organismo para una respuesta de "lucha o huida". De este modo el ritmo cardiaco, la contracción miocárdica y el flujo de sangre aumentan; el flujo de sangre se dirige al músculo esquelético aumentando de forma vigorosa su actividad; el metabolismo celular se incrementa; la glucosa se moviliza desde el hígado al músculo aumentando sus niveles en sangre; y las pupilas y bronquios se dilatan (Hoffman y Taylor, 2001).

La disfunción del eje simpatoadrenal, que tiene como resultado una liberación excesiva de catecolaminas, se ha visto implicada en la patogénesis de la hipertensión esencial genética. Así, se ha descrito que los niveles circulantes de noradrenalina y adrenalina están aumentados en ratas espontáneamente hipertensas (SHR) (Iriuchijima, 1973; Grobecker y col., 1975; Pak, 1981).

Por otra parte, también se ha descrito que en pacientes hipertensos hay una mayor actividad del sistema nervioso simpático (Anderson y col., 1989) y se encuentran aumentadas las catecolaminas circulantes (Goldstein, 1983; de Champlain y col., 1999). Estos niveles altos de catecolaminas circulantes se deben, probablemente, a un aumento de la liberación de catecolaminas desde los nervios simpáticos y desde las células cromafines de la médula adrenal (Arnaiz y col., 1978). De hecho, la mayor liberación de catecolaminas se ha observado en las glándulas suprarrenales (Lim y col., 2002) y en células cromafines aisladas (Miranda-Ferreira y col., 2008; Miranda-Ferreira y col., 2009) de las células de SHRs, comparados con las células de las ratas normotensas (NWRs).

Esta posible disfunción simpática ha constituido durante muchos años la base para el desarrollo de diversos fármacos antihipertensivos que pudieran interferir con dicho eje, entre otros los bloqueadores de los receptores α y β adrenérgicos, la reserpina, la α-metil-l-dopa, la guanetidina o los agentes bloqueantes ganglionares (Westfall y Westfall, 2010).

1.2.-LA MÉDULA ADRENAL

La médula adrenal forma parte del sistema nervioso simpático. Está inervada por fibras nerviosas colinérgicas preganglionares procedentes de los nervios esplácnicos. Sin embargo, las células cromafines que forman su parénquima no emiten axones hacia ningún órgano efector sino que son células poligonales agrupadas en acinos, rodeadas de capilares. Cuando se estimulan, liberan por exocitosis catecolaminas directamente al torrente sanguíneo. Su efecto, por lo tanto, no está localizado en una sola sinapsis sino que en unos pocos segundos alcanza prácticamente todas las células del organismo. La liberación de catecolaminas adrenomedulares está directamente controlada por el sistema nervioso central.

La médula adrenal (Figura 3) constituye en el hombre alrededor del 30% de la masa total de la glándula suprarrenal, es de origen neuroectodérmico y está constituida principalmente por células cromafines. Las células cromafines están intercaladas por células sustentaculares, consideradas como componentes neurogliares de la médula adrenal; son pequeñas e irregulares y contienen el marcador glial S-100 (Cocchia y Michetti, 1981). Por último la glándula presenta una gran cantidad de vasos sanguíneos y de fibroblastos, los cuales forman un estroma de fibras reticulares que rodea a grupos de células secretoras (Coupland y col., 1964).

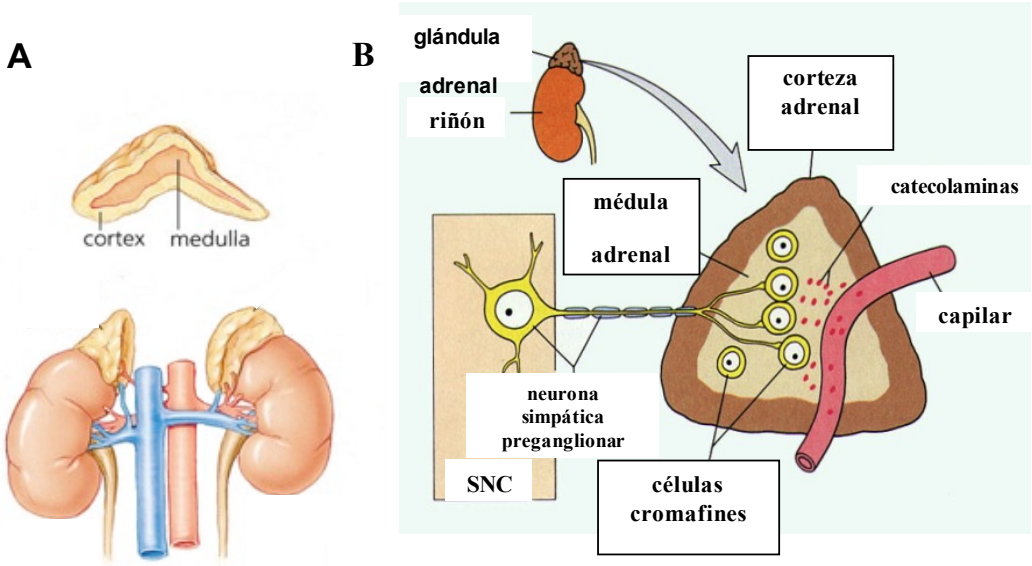

Figura 3. La glándula adrenal. A) Localización, vascularización y estructura de las glándulas adrenales en el organismo humano. **B)** Esquema representativo de la inervación de las células cromafines en el interior de la médula adrenal y de cómo sus productos de secreción (catecolaminas) son vertidos al torrente sanguíneo.

En estudios anatómicos clásicos se encontraron diferencias histoquímicas separando dos poblaciones de adrenalina y noradrenalina que tienen las células cromafines en la medula adrenal (Hillarp y Hokfelt, 1953; Coupland y col., 1964). Hay evidencias que sugieren que estas dos poblaciones de células cromafines son reguladas por distintas vías neurales en la medula adrenal (de Diego y col., 2008). Por lo tanto, estas terminaciones nerviosas en las células secretoras de adrenalina son morfológicamente diferentes a las células secretoras de noradrenalina.

1.2.1.-Secreción diferencial de adrenalina y noradrenalina

La secreción diferencial de adrenalina y noradrenalina de la médula adrenal está firmemente regulada por mecanismos tanto centrales como periféricos. Por ejemplo, la secreción de adrenalina podría ser modificada selectivamente por una estimulación eléctrica de sitios específicos en el córtex del cerebro (Von Euler y Folkow, 1958), en el hipotálamo (Robinson y col., 1983) y la médula oblongada (Lindgren y col., 1959; Matsui, 1965). Por otra parte, la secreción de noradrenalina se ve selectivamente mejorada por la estimulación eléctrica de otros sitios del hipocampo (Folkow y Von Euler, 1954; Robinson y col., 1983) y la médula oblongada (Matsui, 1965).

Esta secreción diferencial de catecolaminas desde la médula adrenal también va a estar condicionada por el estímulo que genera dicha liberación masiva. Así, por ejemplo, en experimentos *in vivo* desarrollados en gatos, la hemorragia causa una liberación preferentemente de noradrenalina, mientras que la hipoglucemia insulínica libera preferentemente adrenalina (Feuerstein y Gutman, 1971). Parece lógico que ante un shock hemorrágico se libere preferentemente noradrenalina porque se requiere una acción vasoconstrictora mediada por receptores alfa-adrenérgicos; por el contrario, la hipoglucemia causa una secreción simpática mediada esencialmente por adrenalina (Khalil y col., 1986) porque moviliza glucosa del hígado y del músculo mediada a través de un mecanismo de receptores beta-adrenérgicos.

En estudios similares en la rata cuando se induce una hipoglucemia por insulina se produce una secreción preferencial de adrenalina (Sun y col., 1979; Gagner y col., 1985; Medvedev y col., 1990; Scheurink y Ritter, 1993; Vollmer y col., 1997). Por el contrario, la exposición al frío produce una secreción preferentemente de noradrenalina en la rata (Vollmer y col., 1992).

Más allá de la sinapsis colinérgica esplácnico-cromafin, la secreción diferencial de catecolaminas puede estar también regulada por la expresión de distintos subtipos de receptores en la membrana de las células adrenérgicas y noradrenérgicas. Se ha descrito que los agonistas del receptor muscarínico causan una secreción diferencial de adrenalina en la adrenal perfundida de gato (Douglas y Poisner, 1965). Lo mismo ocurre con los receptores H_1 de histamina, cuya activación media la secreción preferencial de adrenalina en animales intactos (Trendelenburg, 1954) y en células cromafines bovinas aisladas (Choi y col., 1993).

1.2.2.-El tono simpático

Una característica sorprendente del sistema nervioso autónomo es que se requiere tan solo una baja frecuencia de estimulación para activar los distintos órganos efectores. En un libro de texto clásico, el Guyton's 'Medical Physiology', se indica que "sólo un impulso nervioso cada pocos segundos es suficiente para mantener un efecto normal simpático o parasimpático, y toda la función ocurre cuando las fibras nerviosas disparan de 10 a 20 veces por segundo". Esto contrasta por ejemplo con la activación de las motoneuronas que estimulan el musculo esquelético, que disparan a frecuencias incluso superiores a 500 impulsos por segundo (Guyton y Hall, 2006a).

Los órganos diana del sistema nervioso simpático van a tener una doble inervación: por una parte, son inervados directamente por los nervios simpáticos, y por otra parte son inervados indirectamente por medio de las catecolaminas liberadas por la médula adrenal. Este doble mecanismo de estimulación simpática proporciona un factor de seguridad, es decir, un mecanismo podría sustituir al otro en el caso de que fallara alguno de los dos. Otro valor relevante de la médula adrenal es la capacidad de la adrenalina y noradrenalina de estimular diferentes estructuras del cuerpo que no tienen inervación directa de fibras simpática. Esto es particularmente importante en el caso de la adrenalina, puesto que aumenta la tasa metabólica en prácticamente todas las células del organismo.

En humanos, la secreción de la médula adrenal en reposo es de 0.2 $\mu g\ Kg^{-1}\ min^{-1}$ para la adrenalina y cerca de 0.05 $\mu g\ Kg^{-1}\ min^{-1}$ para la noradrenalina. Estas cantidades dan lugar a niveles de catecolaminas circulantes (noradrenalina, 253 $pg\ ml^{-1}$; adrenalina, 97 $pg\ ml^{-1}$) que en condiciones basales son suficientes para mantener la presión arterial

en valores prácticamente normales, incluso si fueran eliminadas todas las vías simpáticas que directamente actúan sobre el sistema cardiovascular.

1.2.3.-La célula cromafín

Desde el punto de vista estructural y funcional, las células cromafines constituyen el tipo celular más importante de la médula adrenal y se denominan así por la alta afinidad que tienen sus vesículas secretoras para teñirse con sales de cromo, como el cromato potásico (Coupland y col., 1964), si bien sus características fundamentales son su origen embriológico neuroectodérmico, su inervación simpática preganglionar y su capacidad para sintetizar, almacenar y secretar catecolaminas. Como bien apunta el grupo de Levi-Montalcini en 2012 (Bornstein y col., 2012), la célula cromafín se puede considerar como un modelo de cerebro periférico.

En función de la catecolamina que secreten se distinguen fundamentalmente tres tipos de células, adrenérgicas, noradrenérgicas y dopaminérgicas, las cuales se disponen en grupos de varias células rodeando estructuras vasculares. La diferencia bioquímica entre los dos tipos celulares principales (células adrenérgicas y noradrenérgicas) es debida a la presencia en las células adrenérgicas de una enzima adicional, la feniletanolamina N-metil transferasa (PNMT), que convierte la noradrenalina en adrenalina.

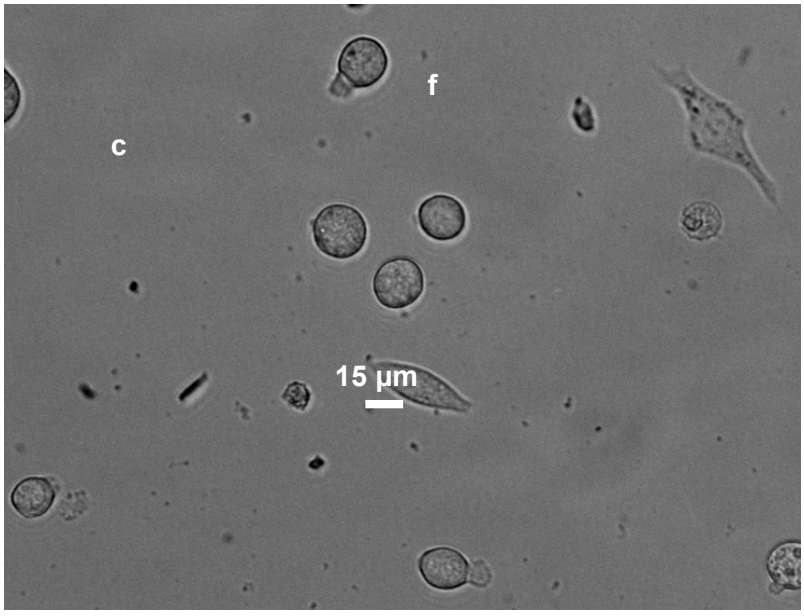

Figura 4. Microfotografía de células cromafines de rata en cultivo.

La morfología de las células cromafines es poligonal y bastante homogénea en el tejido; sin embargo, al cultivarlas se vuelven esféricas, con un diámetro aproximado de 15-20 μm (Figura 4); a nivel de microscopía electrónica la diferencia más llamativa es que las vesículas secretoras de las células adrenérgicas son mayores que las noradrenérgicas, teniendo un contenido de material electrodenso más homogéneo y de menor densidad; por el contrario los gránulos de las células que almacenan noradrenalina poseen un núcleo electrodenso que a menudo ocupa una posición excéntrica (Fawcett y col., 2002). También se ha descrito un tercer tipo de célula cromafín: las células cromafines de vesículas pequeñas, llamadas SIF (Small Intensely Fluorescent cell) (Kanno, 1998) que poseen un fenotipo intermedio entre células noradrenérgicas y neuronas (Aunis, 1998).

No todas las células cromafines se encuentran en la médula adrenal; existen pequeños grupos de células epiteloides que dan una reacción cromafín y que pueden encontrarse en el interior de la corteza y en corpúsculos extraadrenales; se denominan paraganglios y se hayan ampliamente diseminados por el tejido retroperitoneal. Los paraganglios están rodeados de una gruesa envoltura de tejido conjuntivo y en su interior se distinguen dos tipos de células parenquimatosas: las células principales (que poseen numerosos gránulos electroopacos conteniendo catecolaminas) y las células de sostén que rodean a las anteriores (Fawcett y col., 2002).

La médula adrenal se inerva por fibras preganglionares simpáticas procedentes de la médula espinal, cuyos cuerpos celulares se localizan en las columnas celulares intermediolaterales, entre las regiones T3 y L3 (Blaschko y Muscholl, 1972); la mayor parte de estas fibras proceden del nervio esplácnico mayor. También contribuyen a su inervación los nervios esplácnicos menores y otras fibras procedentes de los ganglios celíacos lumbares (Holets y Elde, 1982; Appel y Elde, 1988).

Estas fibras son colinérgicas y en algunos casos se ha descrito la presencia de encefalina como transmisor (Holgert y col., 1995). Todas las fibras inervan tanto a células adrenérgicas como a noradrenérgicas, a excepción de las fibras encefalinérgicas, que inervan exclusivamente a las células adrenérgicas, por lo que se postula que estas fibras pudieran estar implicadas en el control de la secreción de adrenalina (Pelto-Huikko y col., 1985; Holgert y col., 1995).

Los axones pueden contactar con una o varias células, habiéndose observado de 1 a 4 botones sinápticos por célula, en el caso de las células cromafines de rata (Kajiwara y col., 1997).

Las bases anatómicas de los circuitos neurales que permiten al cerebro estimular selectivamente las células adrenérgicas o noradrenérgicas no se conocen, ni tampoco las vías neurales descendentes desde centros supraespinales a la médula espinal. Se piensa que estas dos poblaciones de células secretoras de catecolaminas se pueden estimular de modo preferencial dependiendo del tipo de estímulo estresante, ya sea a nivel de célula individual o sobre grupos de las mismas. Adicionalmente a esta estimulación diferencial, la secreción es modulada por neurotransmisores, neurohormonas u hormonas circulantes, ya que las células cromafines poseen, además de los receptores colinérgicos, una gran variedad de receptores que permiten dicha modulación de la secreción.

1.2.3.1.-La célula cromafín como modelo de neurona secretora

Las células cromafines de la glándula adrenal de mamíferos presentan un gran parentesco con las neuronas simpáticas postganglionares, tanto por su origen embrionario (ambas derivan de la cresta neural) como por su principal función (liberan catecolaminas en respuesta a la estimulación con ACh).

Ambos tipos celulares comparten además otras características funcionales: así, la célula cromafín posee receptores nicotínicos (Douglas y Rubin, 1961b; Wilson y Kirshner, 1977) y muscarínicos (Douglas y Poisner, 1965) sobre los que actúa la ACh; disparan potenciales de acción (Biales y col., 1976; Brandt y col., 1976; Kidokoro y Ritchie, 1980), poseen canales de Ca^{2+}, Na^{+} y K^{+} sensibles a voltaje (Fenwick y col., 1982b; Artalejo y col., 1993; Garcia y col., 2006), y exhiben facilitación de la corriente de calcio por prepulsos despolarizantes repetidos (Ikeda, 1991; Gandia y col., 1993b; Albillos y col., 1996b).

Las células cromafines también presentan similitudes morfológicas con las neuronas postganglionares simpáticas: emiten prolongaciones al co-cultivarlas con astroglia (Uceda y col., 1995) o al exponerlas al factor de crecimiento nervioso (Unsicker y col., 1980).

1.2.3.2.-Funciones fisiológicas de la célula cromafín

Como ya hemos comentado, la principal función de las células cromafines de la médula adrenal va a ser la liberación de las catecolaminas hacia la circulación sistémica en respuesta a distintos estímulos. Estos estímulos secretagogos pueden provenir de un incremento de la actividad de los nervios esplácnicos, que liberan el neurotransmisor fisiológico acetilcolina (ACh), o bien puede tratarse de estímulos químicos que llegan a la médula adrenal por vía hemática. Entre los primeros figuran las situaciones de miedo y ansiedad, la hipoglucemia, la hipotensión o la hipotermia. Por vía hemática van a llegar sustancias como la histamina, la bradikinina o la angiotensina II, que pueden ser liberadas en situaciones como la alergia o la hipotensión.

Las catecolaminas liberadas por las células cromafines pueden actuar sobre diferentes dianas, principalmente localizadas en el sistema cardiovascular, el sistema endocrino y el sistema nervioso central, produciendo un incremento en la frecuencia y fuerza de contracción cardíacas, elevando la presión sanguínea y favoreciendo la liberación hepática de glucosa. Todos estos procesos, junto a la estimulación simpática del corazón y músculos, preparan al organismo para afrontar rápidamente una actitud de huida o de lucha frente al estímulo estresante, como ya se comentó anteriormente.

1.2.3.3.-Canales iónicos en la célula cromafín

Los canales iónicos involucrados en la excitabilidad de las células cromafines han podido estudiarse gracias al desarrollo de las técnicas de *patch-clamp* y de biología molecular y a la aparición de herramientas farmacológicas, fundamentalmente toxinas (procedentes de caracoles marinos, arañas, abejas, alacranes, serpientes, ranas, plantas, etc.) o moléculas orgánicas de síntesis que son capaces de reconocer selectivamente los diversos tipos de canales iónicos presentes en la membrana celular.

Gracias a estas herramientas, en la célula cromafín se han identificado más de 15 tipos distintos de canales iónicos, que pueden dividirse en tres grandes grupos en función del mecanismo que induce o favorece su apertura, distinguiéndose por tanto:

1) **Canales iónicos regulados por voltaje**: Dentro de la superfamilia de canales dependientes de voltaje presentes en la célula cromafín se incluyen los canales de Na^+ (que sirven como mecanismo amplificador de la estimulación nicotínica en respuesta a bajas concentraciones de ACh (Fenwick y col., 1982b)), los de K^+

(que contribuyen a la hiperpolarización/repolarización de ésta durante los potenciales de acción, regulando su patrón de disparo y la exocitosis (Gonzalez-Garcia y col., 1993; Lara y col., 1995; Montiel y col., 1995) y de los que se han descrito principalmente tres tipos, a saber: el canal de K^+ rectificador retrasado, sensible a voltaje, y otros dos activados no sólo por voltaje sino principalmente por calcio intracelular, los canales BK (del inglés "*big conductance"*) (Marty, 1981; Marty y Neher, 1985) y los canales SK (del inglés "*small conductance"*) (Artalejo y col., 1993) y los canales de Ca^{2+} dependientes de voltaje, de los que hablaremos con mayor detalle en el siguiente apartado.

2) **Canales iónicos activados por ligando**: este tipo de canales se abre tras reconocer a una sustancia química específica (su ligando), que puede ser un neurotransmisor o una hormona. En la célula cromafín se han descrito el receptor para la ACh de tipo nicotínico neuronal (Wilson y Kirshner, 1977; Fenwick y col., 1982a; Criado y col., 1992; García-Guzmán y col., 1995; Campos-Caro y col., 1997; Criado y col., 1997), no selectivo para cationes, y el receptor de GABA de tipo $GABA_A$, permeable a Cl^- (Kataoka y col., 1986; Peters y col., 1989).

3) **Canales iónicos operados por depósitos intracelulares**: estos canales constituyen la principal vía de entrada de calcio en las células eucariotas no excitables. Se activan por el vaciado de depósitos intracelulares de calcio (aunque el mecanismo exacto que los activa no está claramente dilucidado) generando una "entrada capacitativa de Ca^{2+}" o "entrada de Ca^{2+} operada por depósitos" destinada a rellenar tales depósitos (Parekh y Putney, 2005). La corriente mejor caracterizada de este tipo es la denominada I_{CRAC} (del inglés "*calcium release activated calcium current"*), una corriente de calcio no activada por voltaje y con rectificación de entrada; se ha descrito en numerosos tipos de células, principalmente de origen hematopoyético. En neuronas y células cromafines bovinas existen evidencias de que se produce entrada de calcio operada por depósitos, aunque no del tipo I_{CRAC} (Fomina y Nowycky, 1999; Putney, 2003).

   Aunque el rellenado de los depósitos de Ca^{2+} pueda parecer la principal misión de estos canales, en realidad juegan un papel fundamental en fenómenos tan variados como los de oscilaciones del calcio citosólico, regulación de la actividad de algunos enzimas y de la transcripción de algunos genes, reacción acrosómica o exocitosis en células no excitables como mastocitos. En células cromafines

bovinas, el grupo de Nowycky (Fomina y Nowycky, 1999) demostró que estas corrientes de entrada de Ca^{2+} son capaces de estimular la exocitosis a potenciales negativos (cuando los CCDV están inactivos) así como de potenciar las respuestas secretoras tras la activación de los CCDV. Se especula que tras un tren de potenciales de acción que deplete de forma considerable la población de vesículas listas para ser secretadas, la entrada de calcio operada por depósitos podría contribuir a la recuperación del tamaño de dicha población ya que, como se sabe, el movimiento de vesículas desde la población de reserva hasta la de las listas para ser secretadas es dependiente de Ca^{2+} citosólico.

1.2.3.4.-Canales de Ca^{2+} dependientes de voltaje (CCDV) en la célula cromafín

Los CCDV son la principal vía de entrada de Ca^{2+} extracelular en la célula cromafín. Su apertura permite la entrada en la célula de grandes cantidades de Ca^{2+} debido al gran gradiente electroquímico existente para el Ca^{2+} entre uno y otro lado de la membrana plasmática.

Los CCDV pueden dividirse, desde un punto de vista biofísico, en dos grandes grupos (Carbone y Lux, 1984; Nowycky y col., 1985; Fox y col., 1987) en función del voltaje requerido para su apertura: los canales de bajo umbral de activación (**LVA**, del inglés "*low voltage-activated"*), que se abren a potenciales por encima de -50 mV, y los canales de alto umbral de activación (**HVA**, del inglés "*high voltage-activated"*), que requieren una fuerte despolarización de la membrana para abrirse (por encima de -30 mV).

Los CCDV **LVA**, o de tipo T (del inglés "*tiny"* o "*transient"*), se caracterizan por activarse tras ligeras despolarizaciones de la célula y por una rápida cinética de inactivación. Han sido identificados en una gran variedad de células excitables; su principal función es la de generar una actividad eléctrica rítmica en las células, así como facilitar la entrada de calcio a potenciales de membrana más hiperpolarizados (Bean, 1985). En cuanto a las células cromafines, se ha descrito su presencia en células cromafines bovinas (Diverse-Pierluissi y col., 1991) y en células cromafines de rata (Hollins y Ikeda, 1996; Bournaud y col., 2001). Se piensa que este tipo de CCVD estaría principalmente presente en células cromafines inmaduras en desarrollo, en las que sería el responsable de la respuesta de estas células a la hipoxia (Levitsky y Lopez-

Barneo, 2009; Souvannakitti y col., 2010) donde se postula que serían reemplazados por CCDV HVA paralelamente a la maduración del tejido y de la maquinaria exocitótica.

Con respecto a los **canales HVA**, se han descrito hasta cinco subtipos, que se diferencian entre sí por sus propiedades biofísicas y farmacológicas: los subtipos L, N, P/Q y R. En la célula cromafín se expresan todos ellos: los L (Hoshi y Smith, 1987; Bossu y col., 1991; Albillos y col., 1994), los N (Bossu y col., 1991; Artalejo y col., 1992; Albillos y col., 1994), los P (Albillos y col., 1993; Gandia y col., 1993a; Artalejo y col., 1994) y los Q (Lopez y col., 1994; Albillos y col., 1996a). Respecto al canal R, se sabe que la célula cromafín bovina expresa ARNm para la subunidad α_{1E} (Garcia-Palomero y col., 2000), implicada en la formación del poro iónico de este subtipo de canal, y que tanto las células cromafines bovinas como las de ratón presentan una fracción de la corriente de Ca^{2+} total que no se bloquea por ninguno de los bloqueantes selectivos para el resto de subtipos de canales de calcio, la cual se atribuye a canales R.

Los diferentes subtipos de canales HVA en la célula cromafín presentan una densidad relativa muy diferente entre especies (Figura 5) (Garcia y col., 2006). Así, los de subtipo P/Q predominan en la humana y bovina, especies en las que los de subtipo L están muy poco representados; los canales L son los más abundantes en rata, ratón y gato y los de subtipo N abundan en células cromafines de cerdo (Figura 5).

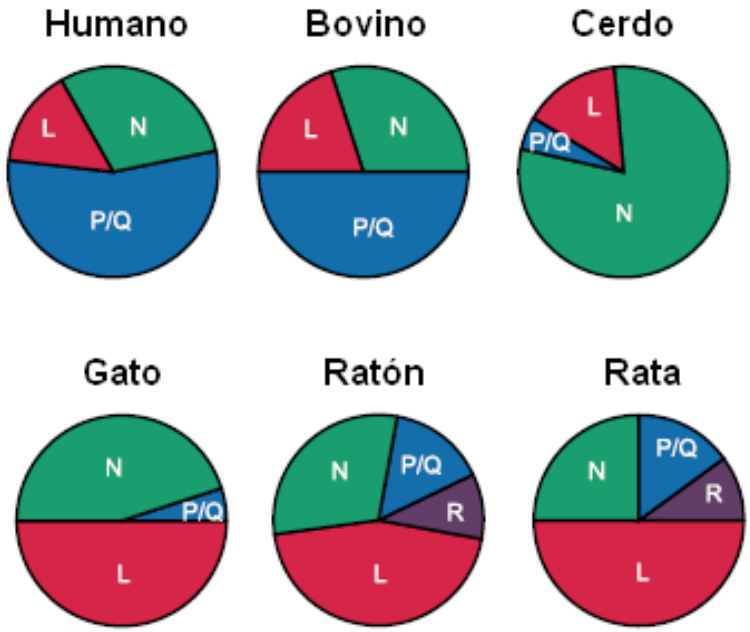

Figura 5. Distribución de los diferentes subtipos de canales de calcio en cultivos primarios de células cromafines de la médula adrenal de distintas especies (Garcia y col., 2006).

También difiere entre especies el grado en que cada uno de estos subtipos de canales HVA contribuye a la secreción de catecolaminas. En células cromafines bovinas los de tipo L y Q parecen ser los principales implicados, (Lopez y col., 1994) e incluso se ha sugerido que los de tipo Q estarían más directamente acoplados a la maquinaria exocitótica que los L (Lara y col., 1998).

1.2.3.5.-Mecanismos moduladores de la actividad de los CCDV

Los CCDV representan un papel crucial en innumerables respuestas celulares y por ello necesitan ser modulados finamente para poder regular las señales citosólicas de Ca^{2+}, evitando así un aumento excesivo de la concentración de Ca^{2+} intracelular que podría llevar a una activación de los mecanismos de muerte presentes en la célula. Dos factores controlan principalmente la actividad de los CCDV: el voltaje y el propio Ca^{2+}. Ambas formas de inactivación son reguladas por diversos dominios en las subunidades α y β (Wykes y col., 2007). También se ha descrito la existencia de mecanismos moduladores autocrino/paracrinos producidos por diversos productos neurosecretores, mediados por proteínas G (Garcia y col., 2006).

Modulación de los CCDV por Ca^{2+}

Los CCDV se pueden modular por una acumulación de Ca^{2+} en la superficie interna de la membrana. Esta inactivación dependiente de Ca^{2+} (CDI, del inglés "*calcium-dependent inactivation"*) se describió por primera vez en la fibra muscular del *Balanus nubilus* por Hagiwara y Nakajima (Hagiwara y Nakajima, 1966). Estos autores observaron que tras aplicar una fuerte despolarización, las contracciones musculares solamente se producían cuando la concentración de Ca^{2+} intracelular se encontraba disminuida por la presencia de quelantes como el EGTA, EDTA o K-citrato. Años más tarde, esta hipótesis fue corroborada con estudios en otras células excitables (Brandt y col., 1976; Brehm y Eckert, 1978; Tillotson, 1979).

Es de destacar que los diferentes subtipos de CCDV se diferencian entre sí en cuanto a su sensibilidad a la inactivación por el Ca^{2+}. Así, los CCDV de tipo L presentes en el músculo esquelético se inactivan rápidamente tras la aplicación de un estímulo despolarizante, mientras que los CCDV neuronales (N, P/Q) presentan una inactivación más lenta (Yue y col., 1990; von Gersdorff y Matthews, 1996).

Algunos autores han descrito que la sustitución por Ba^{2+} en el medio extracelular, así como la reducción de la concentración de Ca^{2+} extracelular y/o la adición de quelantes de Ca^{2+}, ejercen poco efecto sobre la inactivación de los CCDV de tipo N y P/Q, lo que apoyaría la idea de que la modulación de éstos se ejercería solamente por el voltaje (Edwards y Jones, 1993; Patil y col., 1998). Por el contrario, otros grupos han encontrado claras evidencias a favor de la modulación de estos subtipos de CCDV por el Ca^{2+} (Cox y Dunlap, 1994; Tareilus y col., 1994; Lee y col., 1999; Shirokov, 1999). En lo que respecta a los CCDV existentes en la membrana de la célula cromafín, el CCDV tipo L presenta una inactivación dependiente de Ca^{2+} más lenta que la presentada por los canales N y P/Q (Hernández-Guijo y col., 2001).

Modulación de los CCDV por voltaje

Poco se conoce del mecanismo por el cual los CCDV se inactivan por voltaje. Sin embargo, este proceso de inactivación puede ser observado al compararse la corriente generada por una secuencia de dos pulsos de voltaje. Si el intervalo entre los dos pulsos es corto y no permite una recuperación del canal, la inactivación se evidencia como una disminución de la amplitud de la corriente durante el segundo pulso de voltaje (Gutnick y col., 1989). Otra forma de observarse la inactivación por voltaje es cambiando el potencial de fijación de membrana a potenciales menos negativos (Villarroya y col., 1999).

La inactivación por voltaje parece afectar de forma diferente a los distintos subtipos de CCDV y se podría decir que presenta dos fases o estados de inactivación: una inactivación media, a bajos potenciales (en torno a -55 mV), que afecta fundamentalmente al componente sensible a ω-conotoxina GVIA (canales tipo N), y una segunda fase que afecta el componente mantenido de la corriente, inactivado a voltajes sobre -10 mV y que es sensible a dihidropiridinas (canal tipo L). También se ha descrito que los canales del tipo N no sufren inactivación por voltaje (Artalejo y col., 1992). A nivel de la célula cromafín, se ha demostrado que los canales N y P/Q son más susceptibles que los canales L a la inactivación por voltaje (Villarroya y col., 1999).

Modulación de los CCDV por neurotransmisores acoplados a proteínas G

En 1986, se propuso por vez primera que la inhibición de las corrientes de calcio producida por algunos neurotransmisores podría estar siendo modulada por voltaje. En estos experimentos, se observó un enlentecimiento de la activación de los CCDV a potenciales negativos en presencia de dopamina (Marchetti y col., 1986).

En las dos últimas décadas, se ha profundizado en el conocimiento de este tipo de mecanismo modulador de la actividad de los CCDV, habiéndose demostrado el papel de las subunidades βγ de las proteínas G en la inhibición de los CCDV. Así, la unión de diversos neurotransmisores a sus receptores de membrana van a activar determinados subtipos de proteínas G. Las subunidades βγ de la proteína G se unen directamente a la subunidad α_1 del CCDV, haciendo el canal resistente a la apertura (Ikeda, 1996; Fox y col., 2008). Este tipo de regulación de la entrada de Ca^{2+} por la acción de diversos neurotransmisores acoplados a proteínas G se caracteriza por que las corrientes de Ca^{2+} presentan una cinética de activación lenta y una menor inhibición a potenciales positivos. Por otro lado, la aplicación de un breve prepulso despolarizante es capaz de producir una pérdida parcial de la inhibición, lo que ha sido denominado por algunos autores como "facilitación" de la corriente de calcio (Bean, 1985; Ikeda, 1991; Ikeda y Dunlap, 1999; Garcia y col., 2006).

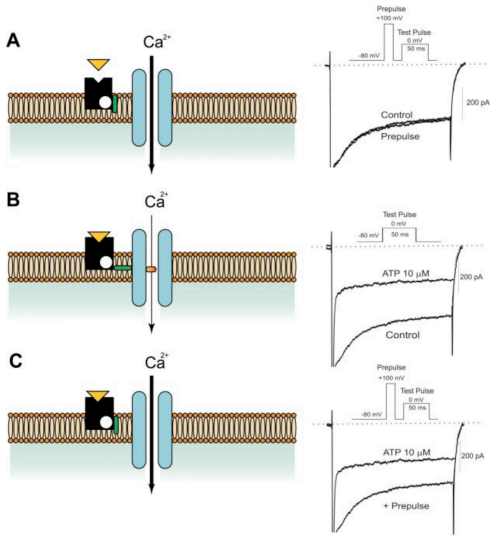

Figura 6: Mecanismo de inhibición de los canales de calcio por proteínas G. En condiciones control, las proteínas G no están acopladas a los CCVD. La aplicación de un estímulo despolarizante, genera una corriente de entrada que no se modifica en caso de la aplicación de un prepulso despolarizante (A). Cuando el ATP se une a los receptores purinérgicos, la proteína G cambia de conformación modulando el canal disminuyendo el pico de la corriente (B). La aplicación de un prepulso despolarizante en estas condiciones desacopla la proteína G del canal de calcio, revertiendo la inactivación (C) (Garcia y col., 2006).

La unión del ATP a su receptor es un ejemplo de la modulación de los CCDV por proteínas G en las células cromafines (Gandia y col., 1993b; Ulate y col., 2000). Cuando el ATP se une a los receptores purinérgicos, se activa una proteína G que actúa enlenteciendo la activación de la corriente de calcio y disminuyendo su amplitud. La aplicación de un prepulso despolarizante va a actuar desacoplando la proteína G del canal, promoviendo así una aparente "facilitación" de la corriente de entrada (Figura 6).

Dos observaciones experimentales apoyan el papel de la proteína G en la modulación de los CCDV. Por un lado, tanto la diálisis celular con GDP-β-S como el pretratamiento con toxina *Pertussis,* ambos inhibidores de la actividad de las proteínas G, impiden la inactivación de los canales por neurotransmisores. Por otro lado, la adición a la solución intracelular de GTP-γ-S, un análogo no hidrolizable de GTP que actúa como activador de la proteína G, inhibe las corrientes de calcio (Diverse-Pierluissi y col., 1991; Gandia y col., 1993b; Garcia y col., 2006; Fox y col., 2008).

A nivel de la célula cromafín, también se ha demostrado que la activación de los receptores opioides μ y δ inhiben la corriente de calcio y disminuyen su activación y que diversos componentes de las vesículas secretoras (catecolaminas, ATP, neuropéptidos y opioides) podrían estar modulando los CCDV a través de este mecanismo mediado por proteínas G, habiéndose descrito que se trataría de un mecanismo de modulación de tipo autocrino/paracrino de los CCDV (Albillos y col., 1996a; Garcia y col., 2006).

1.2.2.6.-Ciclo de actividad de una célula cromafín

Ya en 1934 se sugirió que la ACh es el principal agonista fisiológico de las células cromafines (Feldberg y col., 1934). Ésta es liberada por las fibras del nervio esplácnico, que en condiciones de reposo se estima que disparan de forma asincrónica con una frecuencia de 0,1 Hz (Biales y col., 1976; Brandt y col., 1976); en situaciones de estrés, sin embargo, su frecuencia de descarga se incrementa hasta tres veces y puede llegar a sincronizarse, lo cual se traduce en la liberación de gran cantidad de ACh al espacio sináptico esplácnico-cromafín. En 1980, Kidokoro y Ritchie describieron que el grado de liberación de catecolaminas por la célula cromafín se incrementa de forma dosis-dependiente con la frecuencia de disparo de potenciales de acción inducidos en ésta por la ACh (Kidokoro y Ritchie, 1980); fue la primera evidencia clara del

acoplamiento entre el comportamiento eléctrico de la célula cromafín y su función secretora.

La ACh liberada por el nervio esplácnico activará entonces receptores nicotínicos y/o muscarínicos presentes en la membrana de la célula cromafín; aunque el porcentaje de uno y otro colinoceptor varía entre especies animales y se altera en situaciones de estrés crónico, en los mamíferos predomina la acción de la ACh sobre los receptores nicotínicos (O'Sullivan y Burgoyne, 1989).

Tras la unión de la ACh al receptor nicotínico se va a producir la apertura de su canal iónico, penetrando a su través iones Na^+ y, en menor medida, Ca^{2+} (Figura 7) (Douglas y col., 1967) . La entrada de Na^+ va a provocar una ligera despolarización de la membrana, suficiente para inducir la apertura de canales de Na^+ voltaje dependientes (Gonzalez-Garcia y col., 1993). La apertura de estos canales de Na^+ conduce a la entrada masiva de Na^+ al interior celular, lo que va a favorecer una mayor despolarización celular, capaz de inducir la activación de los canales de Ca^{2+} voltaje-dependientes y con ello la entrada de Ca^{2+} desde el espacio extracelular (Garcia y col., 1984). Paralelamente, la ACh puede activar receptores muscarínicos, cuyos efectos en la excitabilidad de la célula y en la secreción de catecolaminas van a ser muy variables entre especies.

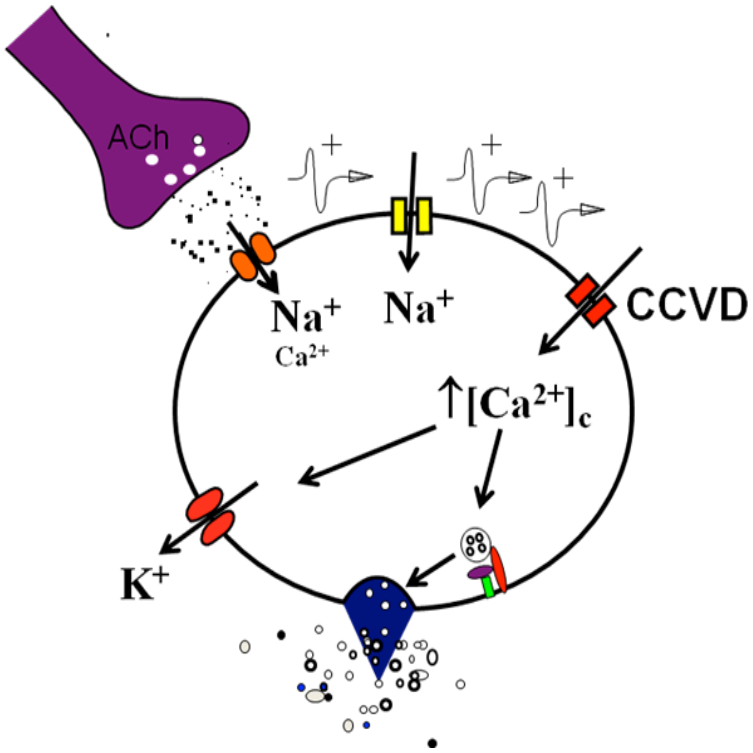

Figura 7. Esquema del proceso de activación de una célula cromafín

Durante todo este proceso de activación celular, la mitocondria y el retículo endoplásmico estarán también activos, ejerciendo un riguroso control de los transientes de $[Ca^{2+}]_c$ que se alcanzan en diferentes zonas del citosol, y especialmente de los microdominios del calcio que se alcanzan en las regiones subplasmalemales, relacionadas con los sitios de exocitosis.

La finalización del ciclo de actividad de la célula cromafín se puede producir por varios mecanismos:

1) hidrólisis de la ACh –a colina y acetato- en el espacio sináptico por parte de la enzima acetilcolinesterasa.
2) desensibilización de los receptores nicotínicos por exposición continuada a ACh.
3) inactivación de los CCDV por el propio Ca^{2+} y por el voltaje.
4) inhibición de los CCDV por productos de secreción (ATP y opioides).
5) activación de los canales de K^+ dependientes de Ca^{2+}, que favorecen la repolarización de la membrana plasmática, disminuyendo la excitabilidad celular.

1.3.-HOMEOSTASIA CELULAR DEL CALCIO

El Ca^{2+} puede considerarse un **mensajero intracelular universal** que interviene en procesos tan variados como la fecundación del ovocito por el espermatozoide, el desarrollo embrionario, la contracción de los músculos, la coagulación de la sangre, la excitabilidad neuronal, el transporte axoplásmico, la secreción de hormonas o la liberación de neurotransmisores; por otra parte, también está implicado en los procesos de envejecimiento y muerte neuronal como se puede ver en la Figura 8.

La concentración de Ca^{2+} citosólico ($[Ca^{2+}]_c$) en la célula en reposo es bajísima (del orden de 50-200 nM, unas 10.000 veces menor que la concentración extracelular), lo que da lugar a que incrementos relativamente pequeños de Ca^{2+} puedan generar una elevada relación $[Ca^{2+}]_c/[Ca^{2+}]_{c\ reposo}$ y servir de señal que dispara esta gran variedad de fenómenos fisiológicos. Además, el calcio se une con avidez a muchas proteínas, lo que le confiere especial eficacia como segundo mensajero. Por otra parte, este catión se diferencia de otros segundos mensajeros en que no puede sintetizarse *de novo* ni degradarse; las señales intracelulares de Ca^{2+} son el resultado de una regulación rápida y

dinámica entre el Ca^{2+} que entra a través de los CCDV, el Ca^{2+} tamponado, la salida o bombeo de Ca^{2+} desde el citosol hacia el exterior celular y el secuestro/liberación de Ca^{2+} por las organelas intracelulares, regulación que definirá la magnitud, distribución espacio-temporal y frecuencia de la señal citosólica de Ca^{2+}; y que posibilita generar incrementos locales de $[Ca^{2+}]_c$, y por tanto respuestas locales, sin que se produzcan elevaciones globales del $[Ca^{2+}]_c$ en la célula, que implicarían un mayor gasto energético para restaurar los niveles basales de Ca^{2+}.

Una característica del Ca^{2+} como segundo mensajero es su lenta difusión en el citosol, como consecuencia del tamponamiento que sufre al introducirse en la célula. En células endocrinas se estima que sólo uno de cada 30-100 iones de Ca^{2+} se encuentra en estado libre; el resto permanecen reversiblemente unidos a ciertas proteínas que unen Ca^{2+}, como la parvalbúmina, la calbindina o la calmodulina (Neher y Augustine, 1992). Estas proteínas actúan como primer mecanismo de tamponamiento tras la entrada masiva de Ca^{2+}, pero la célula cromafín presenta además otros mecanismos que evitarán su sobrecarga con Ca^{2+} y le permitirán recuperar los niveles de $[Ca^{2+}]_c$ basales:

1) las bombas e intercambiadores de la membrana plasmática, que expulsarán Ca^{2+} hacia el exterior celular
2) las organelas intracelulares dinámicas, como el retículo endoplásmico y la mitocondria, que lo secuestrarán o lo liberarán en función de su estado de rellenado y de la $[Ca^{2+}]_c$
3) las organelas estáticas como las vesículas cromafines, que lo quelarán en su interior.

Existen igualmente unos **mecanismos de salida** (bombas e intercambiadores de Ca^{2+}) y de **secuestro/liberación de Ca^{2+}** (en retículo endoplásmico y mitocondria) que contribuyen en la célula cromafín a la homeostasia del mismo, esquematizados en la Figura 8 (para una revisión más extensa se puede consultar (Berridge y col., 2003)).

1.3.1.-El *retículo endoplásmico (RE)*

El RE es una organela con alta capacidad para almacenar Ca^{2+} y para intercambiarlo rápidamente con el citosol. La $[Ca^{2+}]$ en el interior del RE ($[Ca^{2+}]_{RE}$) se

ha estimado en aproximadamente 5-10 mM. Pero la mayoría de este Ca^{2+} está unido a calreticulina y a otras proteínas que unen Ca^{2+}, de modo que los valores de $[Ca^{2+}]_{RE}$ en forma libre se estiman en un rango que va desde µM hasta 3 mM, según las técnicas empleadas (Meldolesi y Pozzan, 1998).

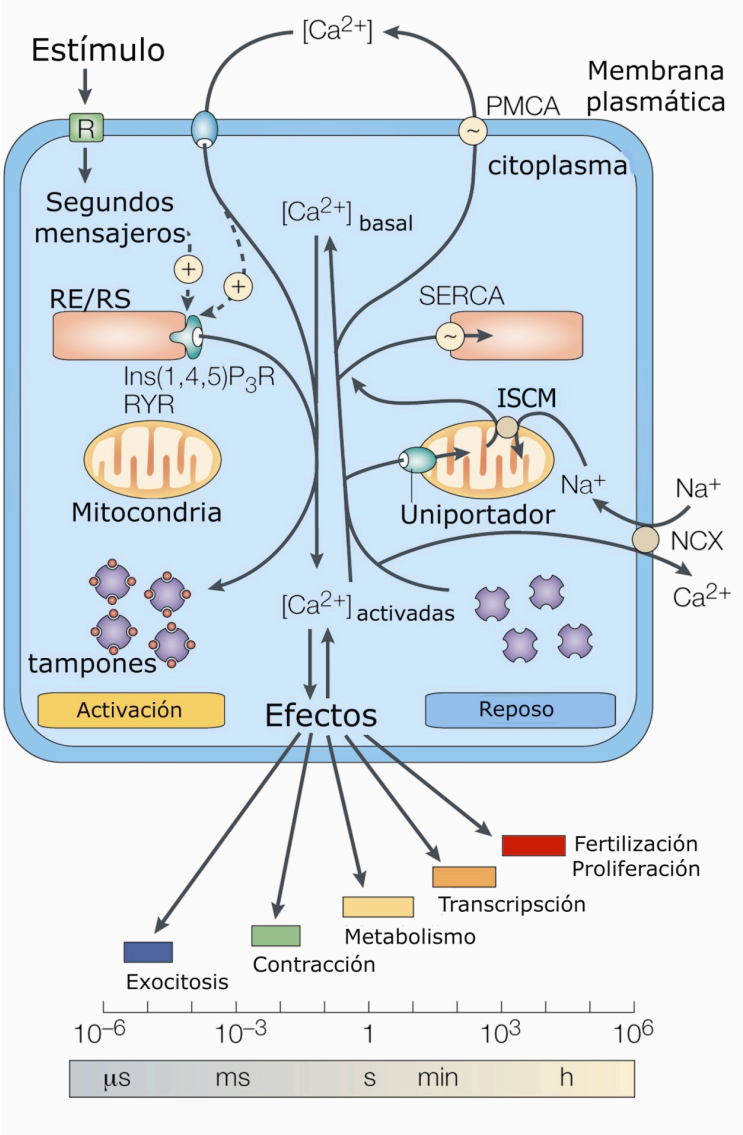

Figura 8. Resumen de los principales mecanismos de señalización y de homeostasia del calcio en una célula modelo (adaptado de Berridge y col., 2003).

En la membrana del RE de la célula cromafín se encuentran tres estructuras que participan en la homeostasia del calcio:

a) una **bomba ATPasa de Ca^{2+}** (la **SERCA**, del inglés "*Sarcoplasmic and Endoplasmic Reticulum Ca^{2+} ATPase*"). La SERCA toma el Ca^{2+} del citosol y lo acumula en el lumen del RE en contra de gradiente electroquímico, con gasto de

ATP (Pozzan y col., 1994; Meldolesi y Pozzan, 1998). Esta bomba posee una baja capacidad pero una alta afinidad por el Ca^{2+} (0,1-1 μM), por lo que su función principal es la de controlar la $[Ca^{2+}]_c$ en condiciones de reposo o en ausencia de estímulo.

Uno de los fármacos más empleados en investigación para depletar el RE de Ca^{2+} es la tapsigargina, un inhibidor irreversible de la SERCA que utilizamos en nuestro estudio (Thastrup y col., 1990).

b) los **receptores de rianodina** (RyR). Se encuentran presentes en casi todos los tipos celulares de mamíferos. En condiciones fisiológicas se activan sólo por Ca^{2+} (Zucchi y Ronca-Testoni, 1997).

La función de los RyR donde se encuentra mejor descrita es en las células del músculo cardíaco, en las que median el denominado **CICR** ("*Calcium-Induced Calcium Release"*), un fenómeno de amplificación de la señal de Ca^{2+} caracterizado por primera vez por Fabiato (Fabiato, 1983): el Ca^{2+} que entra por los CCDV (de tipo L) activa la salida de Ca^{2+} del RE a través de los RyR, amplificando y propagando la señal de calcio por el citosol. La liberación de Ca^{2+} por CICR responde a una curva en campana. Al comienzo, la liberación se incrementa por un mecanismo de retroalimentación positiva; pero al alcanzarse elevadas $[Ca^{2+}]_c$ la liberación de Ca^{2+} se inhibe (con una CI_{50} de 120-150 μM) (Meissner y col., 1986).

La rianodina, compuesto que da nombre a los RyR y que también utilizamos en nuestro estudio, modifica la actividad de éstos de diferentes maneras en función de su concentración. Así, en el rango nanomolar los activa; en el rango de decenas de micromolar los deja, de forma uso-dependiente, en un estado de semiconductancia caracterizado por un tiempo medio de apertura de hasta 20 veces mayor y por insensibilidad a la activación por Ca^{2+}; finalmente, a concentraciones por encima de 200 μM, bloquea RyR de forma irreversible (Buck y col., 1992; Zucchi y Ronca-Testoni, 1997).

Otro de los compuestos capaz de liberar Ca^{2+} del RE a través de RyR es la cafeína (Cheek y col., 1993), hasta el punto de que en ausencia de calcio extracelular puede provocar la secreción de catecolaminas en muchas especies animales. La actividad de este compuesto sobre RyR es dependiente de la $[Ca^{2+}]_{RE}$, de modo

que a concentraciones submaximales la cafeína no puede vaciar por completo el RE de Ca^{2+}, sólo puede liberar Ca^{2+} del RE de forma cuantal, hasta que se alcanzan ciertos niveles de $[Ca^{2+}]_{RE}$ (Alonso y col., 1999).

En células cromafines bovinas, mediante el uso de ecuorinas dirigidas a RE, que permiten monitorizar directamente los cambios en la $[Ca^{2+}]_{RE}$, se pudo demostrar la presencia del fenómeno de CICR en respuesta a pulsos de cafeína, de alto K^+ (70 mM) o del agonista nicotínico DMPP (Alonso y col., 1999). Previamente se contaba sólo con evidencias indirectas del fenómeno: la secreción de catecolaminas en respuesta a estímulos de alto K^+ se veía disminuida tras depletar el RE con cafeína, mientras que se recuperaba conforme se lavaba la cafeína y se rellenaba el RE con pulsos sucesivos de K^++Ca^{2+} (Lara y col., 1997). En este trabajo se sugería la hipótesis de que el RE, en función de su estado de rellenado, podía actuar como una fuente (si está lleno) o un sumidero (si está vacío) de Ca^{2+} citosólico, modulando de este modo la señal de $[Ca^{2+}]_c$ y la secreción de catecolaminas inducidas por la apertura de CCDV.

Con estos tres compuestos: cafeína, rianodina y tapsigargina, hemos elaborado un cóctel que llamamos CRT para depletar de Ca^{2+} el RE e impedir el rellenado del mismo (ver materiales y métodos).

c) los **receptores de inositol 1,4,5-trifosfato** (RIP_3). Estos receptores se encuentran en todos los tipos celulares. En la cascada de señalización intracelular de determinados agonistas se activa la fosfolipasa C, que hidroliza el fosfatidil inositol 4,5-difosfato (PIP_2) de la membrana a diacilglicerol e IP_3. El IP_3 se liberará al citosol, donde activará la salida de Ca^{2+} del RE a través de los RIP_3.

En células cromafines bovinas los agonistas del receptor de ACh de tipo muscarínico, la bradicinina y la histamina activan este tipo de respuesta (Forsberg y col., 1986; Augustine y Neher, 1992; Alonso y col., 1999), que es capaz de desencadenar exocitosis (Augustine y Neher, 1992). Por tanto, en condiciones fisiológicas, el RE de las células cromafines puede actuar como un reservorio de Ca^{2+} tanto vía RyR como vía RIP_3.

La capacidad de RIP_3 para liberar Ca^{2+} del RE es sensible a las concentraciones de calcio intraluminal y citosólico, de modo que, al igual que RyR, su actividad es también dependiente de Ca^{2+} (y no sólo de la presencia de IP_3). Esta dependencia

del Ca^{2+} se ejerce como una modulación del estado de afinidad del receptor por su agonista IP_3, lo que permite a este receptor estar dinámicamente acoplado a las condiciones del medio citosólico y al estado de rellenado del RE para activarse o inhibirse (Taylor, 1998; Miyakawa y col., 2001; Taylor y Laude, 2002; Berridge y col., 2003). Esta habilidad del Ca^{2+} para regular la actividad de RIP_3 explica la generación de oscilaciones intracelulares de Ca^{2+} en células cromafines de rata (Malgaroli y Meldolesi, 1991; Inoue y col., 2004).

1.3.2.-La mitocondria

La visión clásica de que la principal función de la mitocondria en la célula es la de producir energía mediante la síntesis de ATP cambió radicalmente en la década de los 90, cuando se evidenció que esta organela es además esencial en el control de la señal intracelular del Ca^{2+}, tanto en neuronas (Werth y Thayer, 1994; White y Reynolds, 1997; Pivovarova y col., 1999) como en células cromafines (Herrington y col., 1996; Babcock y col., 1997; Montero y col., 2000; Garcia-Sancho y col., 2012).

En la célula cromafín se ha descrito que la mitocondria puede actuar como un depósito rápido y reversible de Ca^{2+} que tampona y modula los incrementos de $[Ca^{2+}]_c$ que se producen tras una despolarización. En células cromafines bovinas ello se traduce en que la mitocondria es capaz de modular procesos como el de la secreción de neurotransmisores (Montero y col., 2000; Montero y col., 2001; Villalobos y col., 2002), la inactivación de CCDV por Ca^{2+} (Hernández-Guijo y col., 2001) o el desensamblaje de los filamentos de F-actina mediado por PKC, que a su vez regula la magnitud de la respuesta secretora (Cuchillo-Ibáñez y col., 2004). Las principales estructuras que contribuyen a ello son:

a) el **uniportador de Ca^{2+} mitocondrial:** Ubicado en la membrana interna de la mitocondria, actúa gracias al gradiente electroquímico generado por la baja concentración de Ca^{2+} en la matriz mitocondrial ($[Ca^{2+}]_M$), similar a la del citosol, del orden de 100-200 nM (Rizzuto y col., 1993), así como por el potencial de la membrana interna mitocondrial, de entre -150 y -180 mV (Duchen, 1999). El uniportador de Ca^{2+} en las células cromafines presenta una baja afinidad y una alta capacidad para unir Ca^{2+}, activándose en un rango de $[Ca^{2+}]_c$ de 500 nM a 2 µM sin llegar a la saturación. Esto se traduce en que, tras un estímulo, la mitocondria

puede captar Ca^{2+} rápidamente, hasta alcanzarse $[Ca^{2+}]_M$ cercanas al rango de milimolar (Herrington y col., 1996; Montero y col., 2000).

Una vez captado el calcio por la mitocondria, el gradiente electroquímico de este catión se invierte y el Ca^{2+} comenzará a salir lentamente de ésta hasta recuperarse los niveles basales de $[Ca^{2+}]_M$ en 1-2 min, gracias a los:

b) **intercambiadores de Na^+/Ca^{2+}** (NCX) y de **H^+/Ca^{2+}**: Son los dos mecanismos involucrados en la salida del Ca^{2+} mitocondrial tras un estímulo. El NCX introduce en el lumen mitocondrial 3 iones Na^+ por cada ion Ca^{2+} que saca al citosol (Reeves y Hale, 1984); posee baja afinidad y alta capacidad para movilizar Ca^{2+} y es reversible: funciona a favor de gradiente iónico y según la magnitud y polaridad de los potenciales electroquímicos. El intercambiador H^+/Ca^{2+} introduce 2 H^+ por cada Ca^{2+} que saca al citosol y se ha caracterizado principalmente en hígado y riñón (Gunter y col., 2000).

1.3.3.-Mecanismos de salida del Ca^{2+} citosólico al exterior celular

En la membrana celular se localizan dos sistemas que pueden estar implicados en la salida del Ca^{2+} desde el citosol hacia el exterior celular:

a) el **intercambiador Na^+/Ca^{2+}**: Es un transportador capaz de extraer grandes cantidades de Ca^{2+} de la célula a cambio de la entrada de Na^+. Posee una baja afinidad por el Ca^{2+}, de hecho su actividad comienza a ser notable a partir de $[Ca^{2+}]_c$ de unos 250 nM, y permanece activo cuando la $[Ca^{2+}]_c$ ha llegado a 2 μM. Por tanto, participa de manera considerable en la expulsión de calcio del citosol tras un estímulo (Tang y col., 2000). Puede actuar en modo reverso, esto es, bombeando Ca^{2+} hacia el interior celular, en función del gradiente electroquímico de Na^+ (Blaustein y Lederer, 1999).

b) la **bomba de Ca^{2+} dependiente de ATP**: Es una ATPasa Ca^{2+}/H^+-dependiente muy semejante a la SERCA, es decir, posee una baja capacidad para transportar Ca^{2+} pero una elevada afinidad por éste (0,1-1 μM), de manera que se satura fácilmente. Esto indica que puede responder a modestas elevaciones de $[Ca^{2+}]_c$ y que por tanto es útil para mantener bajos los niveles de $[Ca^{2+}]_c$ en condiciones de reposo.

HIPÓTESIS Y OBJETIVOS

2.-HIPÓTESIS Y OBJETIVOS

Se piensa que una alteración de la actividad del eje simpatoadrenal, con un incremento de la liberación de catecolaminas, puede ser una de las bases fisiopatológicas implicadas en la hipertensión arterial (HTA). De hecho, se ha observado que los niveles plasmáticos de adrenalina y noradrenalina se encuentran elevados en ratas espontáneamente hipertensas (SHR), de forma similar a lo que ocurre en los pacientes que sufren HTA esencial. En estudios previos realizados en glándulas adrenales intactas de ratas SHR se ha observado que la liberación de catecolaminas inducida por la aplicación del neurotransmisor fisiológico acetilcolina (ACh) o por concentraciones elevadas de K^+, es mayor en ratas SHR, en comparación con ratas normotensas control. Basándonos en estos datos previos, en la presente Tesis Doctoral queremos caracterizar la liberación de catecolaminas a nivel de célula única utilizando un estímulo fisiológico como la ACh frente a un estímulo despolarizante por alto K^+ comparando las células de ratas control con las hipertensas (SHR).

Por otra parte, hace ya mas de 50 años que Douglas y Rubin acuñaron la expresión "*acoplamiento excitación-secreción*" para resaltar la importancia del Ca^{2+} en el desencadenamiento de la secreción de catecolaminas en la célula cromafín de la glándula adrenal de gato. Estos autores establecieron que el mecanismo de liberación era un proceso Ca^{2+}-dependiente (Douglas y Rubin, 1961a). Posteriormente otros autores han validado este concepto. Así, Baker y Knight hicieron experimentos con células cromafines permeabilizadas y corroboraron que la exocitosis era controlada principalmente por Ca^{2+} y también por ATP intracelular (Baker y Knight, 1978); en 1981, Wakade demostró que la secreción de catecolaminas en la adrenal de rata se abolía en ausencia de Ca^{2+} extracelular (Wakade, 1981); Knight y Kesteven (en 1983) determinaron que es el Ca^{2+} en estado libre y ionizado el responsable de la secreción (Knight y Kesteven, 1983).

Como hemos comentado en el apartado de Introducción, en la célula cromafín una vez que se abren los CCDV, el Ca^{2+} entra en la célula merced a un doble gradiente eléctrico y químico pero, además, el incremento de los niveles citosólicos de Ca^{2+} libre puede también deberse a una liberación del catión desde depósitos ubicados en organelas citoplasmáticas (retículo endoplásmico y mitocondria, fundamentalmente; (Alonso y col., 1999; Montero y col., 2000)). Estos depósitos de calcio pueden servir

también para regular la homeostasia celular del calcio y, por ende, el proceso exocitótico de catecolaminas.

En relación a los distintos elementos implicados en la regulación de la exocitosis por el Ca^{2+}, el año 2006 nuestro grupo postuló la hipótesis de que existiría una unidad funcional compuesta por los CCDV, el RE y la mitocondria que estaría regulando los microdominios de Ca^{2+} necesarios para regular las etapas tempranas y tardías del proceso exocitótico y que se vino a denominar la "**Triada funcional**" (Garcia y col., 2006; Garcia y col., 2012). Por ello, en la presente Tesis Doctoral nos planteamos también estudiar la posible implicación de los elementos que integran esta triada funcional (esto es, los CCDV, el RE y la mitocondria), en la regulación de la secreción de catecolaminas en animales control e hipertensos, utilizando para ello tanto células cromafines aisladas como un modelo más fisiológico basado en el uso de rodajas de glándulas adrenales de ratas control y ratas SHR.

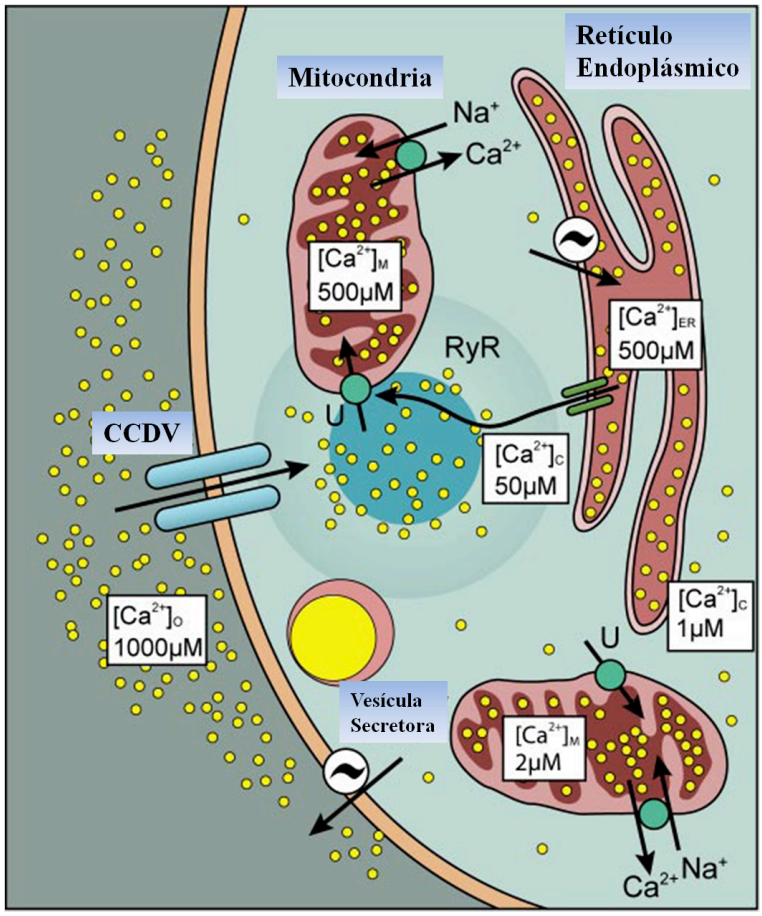

Figura 9: Esquema de la "triada funcional" que regula los movimientos de Ca^{2+} relacionados con la liberación de catecolaminas de la médula adrenal (Garcia y col., 2006).

Basándonos en estos antecedentes e hipótesis de nuestro grupo de investigación, en la presente Tesis Doctoral nos hemos planteado como **objetivo principal** la caracterización del proceso secretor de catecolaminas comparando las células de ratas control con las hipertensas (SHR), así como caracterizar la posible implicación de los diferentes elementos que integran la triada funcional (CCDV, RE y mitocondria), en la regulación de la homeostasia del Ca^{2+} y la consiguiente secreción de catecolaminas en animales control e hipertensos.

Para poder desarrollar este objetivo principal, nos hemos planteado los siguientes **objetivos concretos**:

1. Estudiar la secreción de catecolaminas por métodos amperométricos en célula única utilizando un estímulo fisiológico como la ACh frente a un estímulo despolarizante por alto K^+, comparando las células de ratas control con las hipertensas (articulo 1 de resultados).

2. Estudiar la implicación de las organelas, el RE y la mitocondria, en la secreción estimulando las células, además de los dos anteriores mencionados, con CRT (mezcla de cafeína, rianodina y tapsigargina) para vaciar de calcio el retículo e impedir el rellenado del mismo y FCCP (protonóforo mitocondrial) que vacía de calcio la mitocondria y a su vez impide el rellenado de la mitocondria (articulo 2 de resultados).

3. Estudiar la implicación del RE y la mitocondria en la gestión del calcio en rodajas de glándulas adrenales con sondas fluorescentes específicas para el citosol (Fura 2-AM), para el retículo (Fura FF-AM) y para la mitocondria (Rhod 2-AM) (articulo 3 de resultados).

4. Estudiar la implicación de los distintos subtipos de canales de calcio dependientes de voltaje (CCDV) tanto en rodajas como en célula única con las técnicas de patch-clamp y sondas fluorescentes y su posible modulación por ATP exógeno, comparando las células cromafines control con las hipertensas (articulo 4 de resultados).

MATERIALES Y MÉTODOS

3.-MATERIALES Y MÉTODOS

3.1.-ANIMALES

Para el desarrollo de la parte experimental de la presente Tesis Doctoral se han utilizado ratas machos de 16 semanas de edad, normotensas e hipertensas (cepa SHR; (Iriuchijima, 1973; Grobecker y col., 1975; Pak, 1981), de aproximadamente 300 g de peso, estabuladas en condiciones de 24±2ºC con un 60% de humedad relativa y un ciclo de 12 horas de luz/oscuridad. Los animales fueron alimentados con una dieta estándar y agua *ad libitum.*

Los animales se manipularon de acuerdo a las directrices del Comité de Ética para el manejo de animales de investigación (normas europeas y españolas) de la Facultad de Medicina de la Universidad Autónoma de Madrid.

3.2.-AISLAMIENTO Y CULTIVO DE CÉLULAS CROMAFINES DE RATA

Los animales procedentes del animalario fueron sacrificados por decapitación. Una vez abierto el abdomen y extraídas las glándulas adrenales, éstas se mantuvieron en medio Locke frío y libre de Ca^{2+} y Mg^{2+} con la siguiente composición (en mM) 154 NaCl; 3,6 KCl; 5,6 $NaHCO_3$; 11 glucosa; 10 Hepes; pH 7.2.

Posteriormente, y bajo condiciones de esterilidad, se procedió a la disección manual de la médula adrenal (Figura 10), colocando la glándula sobre un trozo de papel de filtro estéril humedecido en solución de Locke situado sobre una placa de Petri, bajo un microscopio estereoscópico. Primeramente se eliminó toda la grasa periglandular, tras lo cual se realizó un corte de arriba abajo en la cápsula a partir del cual se disecó el interior glandular al arrastrar la hoja de un bisturí hacia el lado opuesto del corte. A continuación y con ayuda del bisturí se recortó todo el tejido cortical, que recubre la médula, disecando esta última completamente (Figura 10).

Los fragmentos de médula se colocaron en un tubo Falcon conteniendo una solución de enzimas digestivas en medio Locke (2 mg/ml de colagenasa, 0,15 mg/ml de DNAsa, y 0,15 mg/ml de hialuronidasa) incubándose en esta solución enzimática durante 20-30 minutos a 37º C. Cada cinco minutos se agitaban suavemente los trozos de médula, ayudándonos de una pipeta pasteur de vidrio. Una vez finalizada la fase de digestión enzimática se disgregaron las médulas, mecánicamente, con ayuda de una

pipeta pasteur, y rápidamente se procedió a parar la reacción enzimática añadiendo a la misma 5 ml de medio con suero bovino fetal. Acto seguido se filtró la solución celular resultante a través de una malla de 80 μm de poro, eliminando de esta manera todos los restos de tejido que no fueron digeridos.

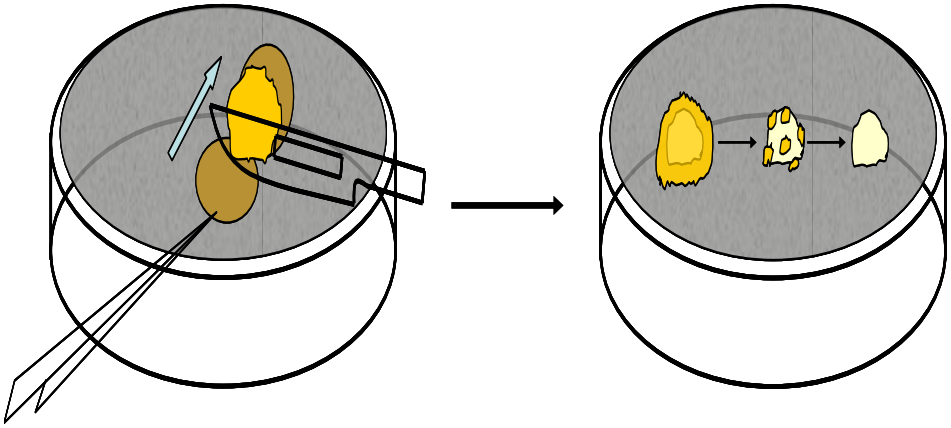

Figura 10. Representación esquemática de la disección de la médula de la glándula adrenal de rata.

Seguidamente y con objeto de eliminar las enzimas digestivas, se centrífugó la solución filtrada a 190xg durante diez minutos, descartando el sobrenadante y resuspendiendo el precipitado con las células en solución de Locke. Después se realizaron dos lavados más mediante la adición de 15 ml de Locke (primer lavado) o medio Eagle modificado por Dulbecco (DMEM; segundo lavado) y su posterior centrifugación (190xg durante 10 minutos).

Después de la segunda centrifugación, el precipitado con las células aisladas se resuspendió en 1-2 ml de DMEM y esta suspensión celular se añadió sobre los cubreobjetos, previamente tratados con polilisina, para facilitar la adhesión de las células, a razón de dos gotas por cubreobjeto. Al cabo de unos 10-15 minutos se añadieron unos 2 ml de DMEM, conteniendo un 5% de suero fetal y antibióticos (50 IU/ml de penicilina y 50 μg/ml de estreptomicina).

Las células se mantuvieron de dos a seis días a 37º C en un incubador con una atmósfera saturada en agua y con un 5% de CO_2. Después de 24 h, el medio se reemplazó por un medio nuevo y posteriormente se cambió cada dos días.

3.3.-REGISTRO AMPEROMÉTRICO DE LA LIBERACIÓN DE CATECOLAMINAS EN CÉLULA ÚNICA

La amperometría es un método electroquímico basado en la oxidación o reducción de sustancias. Muchos productos de secreción, entre ellos las catecolaminas, contienen en su molécula grupos químicos con capacidad para ceder (oxidarse; Figura 11) o captar (reducirse) electrones. La amperometría aprovecha estos fenómenos de óxido-reducción para detectar los productos de secreción por la oxidación de los mismos. Es una técnica de fácil manejo y no invasiva para las células; sin embargo, no permite identificar qué sustancias han sido oxidadas (para ello debería emplearse otro método electroquímico como es la voltametría cíclica).

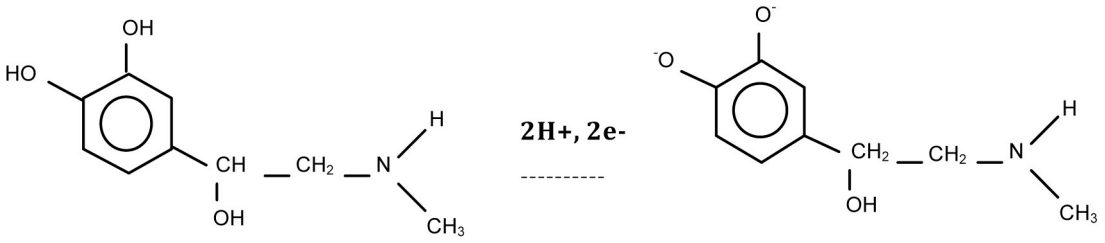

Figura 11. Representación esquemática de la oxidación de una molécula de adrenalina.

La detección por oxidación de las catecolaminas liberadas se consigue mediante un electrodo de carbono; cuando las sustancias oxidables difunden hacia la superficie de este electrodo, los electrones generados en la reacción de oxidación (en el caso de las catecolaminas, 2 e^- por cada molécula oxidada, ver figura 11) serán transferidos a dicho electrodo, produciéndose una corriente eléctrica que será directamente proporcional a la concentración de catecolaminas oxidadas. Es de destacar que cada compuesto de óxido-reducción presenta un estado de equilibrio en el que la mitad de las moléculas se encuentran en estado oxidado y la otra mitad en estado reducido. Dicho estado de equilibrio es consecuencia de un potencial, característico de cada especie química, que recibe el nombre de potencial *redox*. Para favorecer la oxidación de todas las moléculas de catecolaminas secretadas debe aplicarse un voltaje que exceda al potencial *redox* en al menos +200 mV, ya que la aplicación de uno menor disminuiría drásticamente la velocidad de oxidación y por tanto la detección de señales amperométricas. En el caso

de las catecolaminas el potencial utilizado suele ser de +650 a +800 mV (+700 mV en nuestro caso).

En estos experimentos de caracterización de la liberación de catecolaminas en célula única utilizamos una fibra de carbono de 7 μm de diámetro. Esta fibra se introduce en un capilar de polietileno y se sella con una resina específica para este evento (Figura 12).

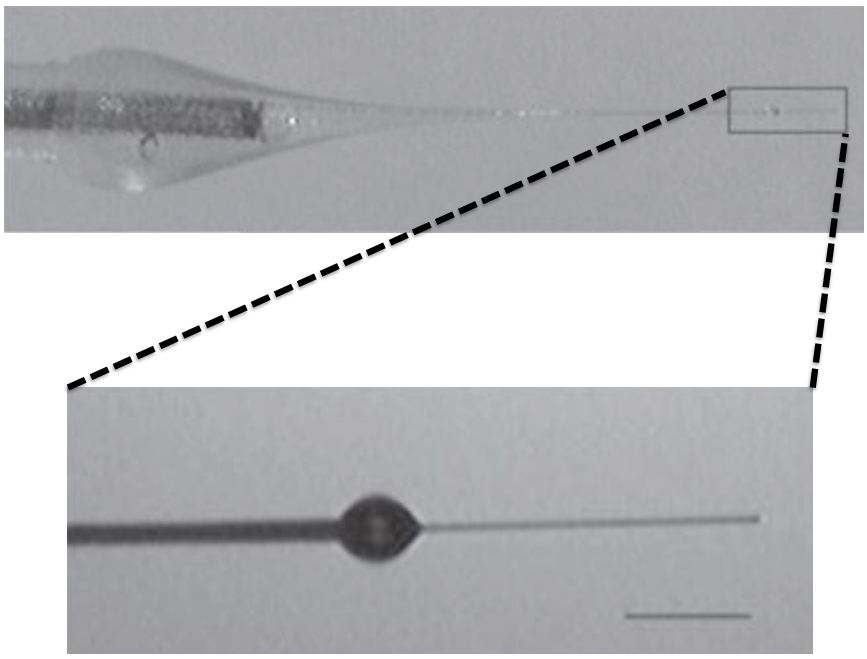

Figura 12. Fotografía de un capilar para registro amperométrico en el que se puede apreciar la fibra de carbono y un detalle pormenorizado de la misma.

El capilar se rellena con una solución de 3 M de K^+ para facilitar la conectividad eléctrica con un electrodo de Ag/AgCl, conectado con un pre-amplificador de *patch-clamp,* conectado a su vez a un amplificador EPC9, que es controlado mediante el programa informático PULSE (desarrollado por la empresa HEKA, Lambrecht/Pfalz, Germany). Para poder detectar la secreción de catecolaminas, como hemos mencionado anteriormente, se utiliza un voltaje de oxidación de +700. Para el registro de los eventos exocitóticos, el capilar que lleva la fibra de carbono con el electrodo de registro, se posiciona en el centro de la célula (ver figura 13).

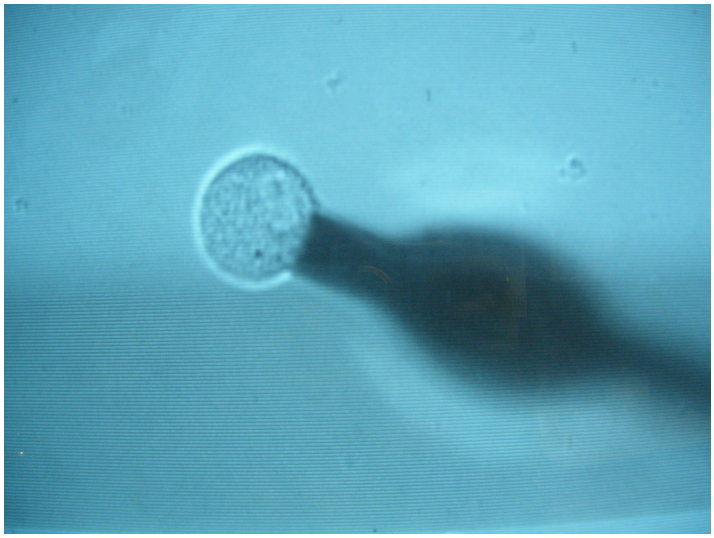

Figura 13. Detalle de aproximación de la fibra de carbono a una célula cromafín.

Para inducir la secreción de catecolaminas se han utilizado distintos agentes secretagogos, según el objetivo del experimento concreto. Generalmente, las células se estimularon con una solución despolarizante conteniendo 70 mM de K^+ o con el neurotransmisor fisiológico ACh. Para la aplicación de los distintos estímulos secretagogos utilizamos una pipeta múltiple, que se aproxima a la célula (posicionada a aproximadamente 40 μm de la célula en experimentación), regulándose la velocidad del flujo de perfusión mediante gravedad y utilizando un ordenador para controlar la apertura-cierre de las electroválvulas que dispensan las distintas soluciones a emplear (Figura 14).

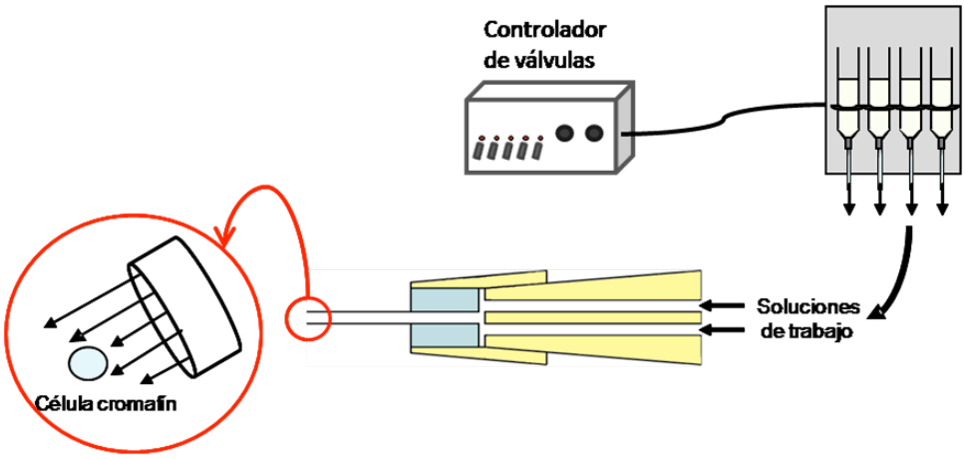

Figura 14. Representación esquemática del sistema utilizado para la perfusión de las distintas soluciones.

Para el análisis de los registros amperométricas hemos utilizado distintos programas informáticos, analizándose diversos parámetros cinéticos de las espigas de secreción, entre otros la amplitud de la respuesta secretora de catecolaminas (medida en pA); la carga total de catecolaminas liberada ("Q", medida en pC); el tiempo que tarda la espiga en alcanzar su amplitud máxima (t_{max}; medido en ms); la pendiente de activación de la espiga amperométrica ("m"; medido en pA/ms); y el tiempo que tarda la espiga en alcanzar la mitad de su efecto total ($t_{1/2}$; medido en ms).

3.4.-MEDIDA DE LOS NIVELES DE Ca^{2+} EN RODAJAS DE GLÁNDULA ADRENAL DE RATA

Una vez extraídas las glándulas del animal, estas se introducen en un liquido nutricio Tyrode frio (Tª 0 a -2ºC) con la siguiente composición (en mM) 137 NaCl; 5,4 KCl; 1,8 $CaCl_2$; 1 $MgCl_2$; 12 $NaHCO_3$; 0,36 KH_2PO_4; 11 glucosa, pH 7.3 ajustado con carbógeno (95% O_2/5% CO_2).

Una vez que se ha desprovisto a la glándula de su cápsula se embebe en una solución de agarosa al 4% de bajo punto de ebullición e inmediatamente se coloca en un vibratomo (1000 Plus Sectioning System, Campden Instruments, INC., U.S.A.) y se realizan cortes de 200 μm (Figura 15).

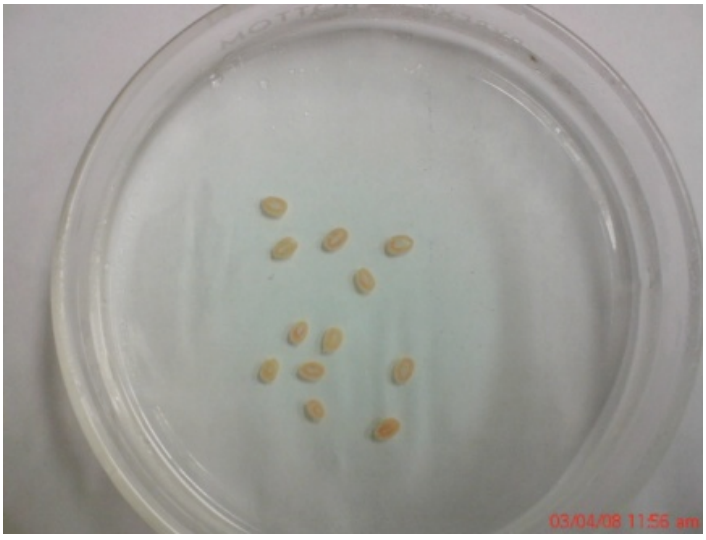

Figura 15. Rodajas de médula adrenal de rata de 200 μm.

Las rodajas obtenidas se incuban con los distintos indicadores fluorescentes de Ca^{2+} dependiendo del objetivo experimental como se describe a continuación:

3.4.1.-Medida de los niveles de Ca^{2+} en el citosol

Para estudiar los cambios de Ca^{2+} en el citosol ($[Ca^{2+}]_C$) se incuban las rodajas de glándula adrenal con la sonda fluorescente Fura 2-AM (Grynkiewicz y col., 1985).

En estos experimentos, las rodajas se incubaron en una solución de Tyrode conteniendo Fura 2-AM (4 μM) y 0,01% de ácido plurónico F-127, a temperatura ambiente durante 45 min, oxigenada con carbógeno (95% O_2/5% CO_2) y protegida de la luz.

Después de este periodo de incubación se lavan las rodajas y se colocan en un microscopio de fluorescencia (TE 300; Nikon, Osaka, Japón) acoplado a una cámara de video (Quantix 512; Roper Scientific Inc., Princeton Instruments, NJ, U.S.A.) y controlado mediante el programa informático Spectralyser (Paul Anderson, NJ, U.S.A.), manteniéndose a una temperatura de 37ºC durante todo el experimento.

El $[Ca^{2+}]_C$ basal se mide durante las 10 primeras imágenes, con un intervalo de 5 s entre cada una de ellas. Las células cromafines de las rodajas de la médula adrenal se estimulan con ACh (100 μM) o con KCl (70 mM) para provocar la entrada de Ca^{2+} a través de los CCDV. Para calcular la fluorescencia máxima de la preparación con Fura 2-AM se incuban las rodajas con digitonina (1 μM) (Miranda-Ferreira y col., 2010).

3.4.2.-Medida de los niveles de Ca^{2+} en el retículo endoplásmico

Para estudiar el Ca^{2+} del retículo endoplásmico, las rodajas se incubaron con la sonda fluorescente Fura FF-AM, que presenta una alta afinidad por el RE cuando se incuba durante largos periodos a 37ºC (London y col., 1994; Hajnoczky y Thomas, 1997; Hajnoczky y col., 1999).

Las rodajas se incubaron en una solución de Tyrode conteniendo Fura FF-AM (10 μM) y 0,01% de ácido plurónico F-127 a 37ºC durante 90 min, oxigenada con carbógeno (95% O_2/5% CO_2) y protegida de la luz.

Después de la incubación con Fura-FF, las rodajas se incubaron con un medio libre de Ca^{2+} y con digitonina (5 μM) durante 30 s con el fin de provocar una semi-permeabilización de la membrana plasmática para facilitar la salida de la sonda fluorescente que no haya sido atrapada dentro del RE. La solución libre de Ca^{2+} (solución intracelular) contiene (en mM): 10 NaCl; 120 KCl; 1 KH_2PO_4; 20 HEPES; y 2

EGTA; esta solución se suplementa con 1 mM de ATP, 10 μM de fosfato de creatina, 10 μM fosfo-creatin-cinasa y 2 mM piruvato, para contrarrestar la energía perdida por la célula al ser semi-permeabilizada. La calibración de la sonda fluorescente se realiza adicionando al medio ionomicina (1 mM), un ionóforo que permea la membrana con el fin de determinar la fluorescencia mínima (R_{min}) al final del experimento (Miranda-Ferreira y col., 2010).

3.4.3.-Medida de los niveles de Ca^{2+} en la mitocondria

Para caracterizar los movimientos de Ca^{2+} en la mitocondria, las rodajas de glándula adrenal se han incubado con la sonda fluorescente Rhod 2-AM, un indicador que presenta una alta afinidad por la mitocondria (Babcock y col., 1997; Golovina y Blaustein, 1997; Hoth y col., 1997).

Las rodajas se incubaron en una solución de Tyrode conteniendo Rhod 2-AM (10 μM) y 0,01% de ácido plurónico F-127 a 37ºC durante 60 min, oxigenada y protegida de la luz.

Después de la incubación con Rhod 2-AM, las rodajas se incubaron en una solución libre de Ca^{2+} con digitonina (5 μM) durante 30 s para producir una semi-permeabilización de la membrana plasmática que permite eliminar la sonda que no haya sido captada por la mitocondria. La solución libre de Ca^{2+} (solución intracelular) está compuesta (en mM): 35 NaCl; 115 KCl; 1 KH_2PO_4; 20 HEPES; y 2 EGTA, pH 7,2; esta solución se suplementa con 1 mM de ATP, 10 μM de fosfato de creatina, 10 μM fosfo-creatin-cinasa y 2 mM piruvato para contrarrestar la energía perdida por la célula al ser semi-permeabilizada. La calibración de esta sonda se realizó adicionando 1 mM de ionomicina de modo que se puede establecer la fluorescencia mínima de la preparación (R_{min}) al final del experimento (Miranda-Ferreira y col., 2010).

Para estudiar los cambios de $[Ca^{2+}]_M$ hemos utilizado el protonóforo mitocondrial FCCP para estimular la salida del Ca^{2+} desde la mitocondria. También hemos estudiado el posible trasiego de Ca^{2+} que pueda haber entre el retículo endoplásmico y la mitocondria con el cóctel CRT para estimular la salida de Ca^{2+} del retículo y observar cómo entra en la mitocondria (ver resultados).

3.5.-REGISTROS ELECTROFISIOLÓGICOS DE PATCH-CLAMP

Para medir las corrientes iónicas que fluyen a través de los canales iónicos de la membrana de las células cromafines se ha utilizado la técnica de registro electrofisiológico de *"patch-clamp"* en su configuración de célula entera (Hamill y col., 1981).

Los cubreobjetos que contienen las células se colocan en una pequeña cámara experimental, montada en un microscopio invertido (Nikon Diaphot). Esta cámara está continuamente perfundida con una solución control de Tyrode (solución extracelular) que contiene (en mM): 137 NaCl; 1 $MgCl_2$; 2 $CaCl_2$; 10 HEPES; pH 7.4. Para registrar las corrientes de entrada a través de los CCDV se ha empleado 10 mM Ba^{2+} como ión transportador de carga (en lugar de 2 mM Ca^{2+}). Las células son dializadas internamente con una solución (solución intracelular) que contiene (en mM): 10 NaCl; 100 CsCl; 20 tetraetilamonio (TEA) Cl; 5 MgATP; 14 EGTA; 20 HEPES; 0,3 NaGTP; a pH 7.2.

El registro de las corrientes se hace mediante un capilar de vidrio pulido al fuego, con una resistencia de unos 2- 5 MΩ, que se adapta a un preamplificador que está conectado a su vez a un amplificador de *patch-clamp* EPC-9 (HEKA Elektronik), que permite la compensación de los transitorios capacitativos y de la resistencia en serie. Tanto la adquisición de los registros como el posterior análisis de los mismos se realiza mediante el programa informático PULSE (HEKA Elektronik). La adquisición de los datos se hace a una frecuencia de muestreo de 10-20 KHz.

Mientras no se indique lo contrario, las células se mantuvieron con un potencial de membrana fijado en -80 mV. Para la activación de los CCDV se aplicaron pulsos despolarizantes a 0 mV (células SHR) o +10 mV (células control) durante 50 ms, con un intervalo de 30 s entre ellos para evitar la pérdida de corriente (Fenwick y col., 1982a).

El intercambio de las distintas soluciones empleadas que bañan a las células se hace a través de una pipeta de perfusión (ver Figura 14). Este sistema facilita el intercambio rápido de las distintas soluciones gracias a un controlador de electroválvulas, accionado por el ordenador. La velocidad de perfusión de las células se regula mediante gravedad, siendo el flujo de 0,5- 1 ml/min, lo cual permite un cambio total de soluciones alrededor de la célula en experimentación en menos de 1 s.

Para la disección farmacológica de los distintos subtipos de CCDV se han utilizado distintas bloqueantes selectivos de éstos, como: nifedipino (3 μM) para bloquear los CCDV del subtipo L; ω-conotoxina GVIA (1 μM) para bloquear los CCDV del subtipo N; y ω-agatoxina IVA (3 μM) para bloquear los CCDV de los subtipos P/Q.

3.6.-DETERMINACIÓN POR PCR CUANTITATIVA DE LOS RECEPTORES PURINÉRGICOS

Para la identificación y cuantificación de los diferentes subtipos de receptores purinérgicos presentes en la membrana de las células cromafines se ha utilizado la técnica de PCR cuantitativa, utilizando ARN obtenido de ratas controles y de ratas SHR.

Para la extracción del ARN total de la médula adrenal de las ratas normotensas y SHR se utilizó Trizol^{TM} (Life technologies, Inc.). Se utilizaron muestras de 1 μg de ARN que fueron tratadas con un amplificador Grade DNase I (Sigma-Aldrich). La transcripción reversa para la síntesis de ADNc se llevó a cabo con un termociclador (Life Technologies, Inc.), usando el sistema de síntesis SuperScript III First-Strand según el protocolo aconsejado por el fabricante (Life technologies, Inc.). La tasa de transcripción de los ARNm seleccionados se determinó utilizando un equipo de PCR cuantitativa en tiempo real (Livak y Schmittgen, 2001) usando el ABI Step One Plus Instrument (LifeTechnologies, Inc.).

La PCR se realizó en 25 μl de una solución tampón que contiene 1 μl de ADNc SYBR Grenn Master Mix (Life technologies, Inc.) y 5 pmol de cebadores específicos para la secuencia de los distintos receptores purinérgicos a explorar y se normalizó comparando GAPDH (Gliceraldehído 3-fosfato deshidrogenasa) con los niveles de ARN de transcripción. Las condiciones de los ciclos en el termociclador consistieron en una preincubación de 2 min a 50ºC, posteriormente una desnaturalización de las muestras a 95ºC durante 10 min, seguido de 35 ciclos de 15 s cada uno a 95ºC y un último ciclo de 1 min a 60ºC. Se utilizó el método comparativo 2-ΔΔCT para la cuantificación relativa de la expresión génica (Livak y Schmittgen, 2001).

Las secuencias de los distintos cebadores empleados en este estudio fueron:

$P2X_1$: F 5`GAGAGTCGGGCCAGGACTTC 3' - R- 5`GCGAATCCCAAACACCTTGA 3';
$P2X_2$: F-5`TCCCTCCCCCACCTAGTCAC 3' - R-5`CACCACCTGCTCAGTCAGAGC 3';
$P2X_3$: F-5`CTGCCTAACCTCACCGACAAG 3' - R-5`AATACCCAGAACGCCACCC 3';
$P2X_4$: F-5`CCCTTTGCCTGCCCAGATAT 3' - R-5`CCGTACGCCTTGGTGAGTGT 3';
$P2X_5$: F- 5`GGATGCCAATGTTGAGGTTGA 3'-R- 5`TCCTGACGAACCCTCTCCAGT 3';
$P2X_6$: F-5`CCCAGAGCATCCTTCTGTTCC 3' - R-5`GGCACCAGCTCCAGATCTCA 3';
$P2X_7$: F-5'GGGAGGTGGTTCAGTGGGTAA 3' - R-5'GGATGCTGTGATCCCAACAAA 3';

$P2Y_1$: F-5`AACCGTGATGTGACCACTGA 3' - R-5` TTCAACTTGTCCGTTCCACA 3';
$P2Y_2$: F-5`TGCTGGGTCTGCTTTTTGCT 3' - R-5`ATCGGAAGGAGTAATAGAGGGT 3';
$P2Y_4$: F-5`TCGATTTGCAAGCCTTCTCT 3' - R-5`CCATAGGAGACCAGGGTGAT 3';
$P2Y_6$: F-5`TGCTGCTACCCCCAGTTTAC 3' - R-5`TGGCATAGAAGAGGAAGCGT 3';
$P2Y_{12}$: F-5`CTGTTTTTTGCTGGGCTCATC 3' - R-5`GCGGATCTGGAAGAAAATCCT 3';
$P2Y_{13}$: F-5`GGATGCAGGGCTTCAACAA 3' - R-5`GCAGCTGTGTCATCCGAGTGT 3';
$P2Y_{14}$: F-5'GGTGGGTTTCGCCTCATGT 3' - R-5'CCTCAGGTGACCGGCATCT 3'.

3.7.-MATERIALES EMPLEADOS

Durante el desarrollo de la parte experimental de la presente Tesis Doctoral se emplearon los siguientes materiales (con indicación de su proveedor habitual):

-La colagenasa tipo I, la ACh, el FCCP, la cafeína, la rianodina, la tapsigargina, el nifedipino y la ADNasa I (Amplificador Grade) se obtuvieron de Sigma (Madrid, España).

-El medio de cultivo DMEM, la albúmina de suero bovino (Fracción V), el suero bovino fetal y los antibióticos se obtuvieron de Gibco (Madrid, España).

-El Fura 2-AM, el Fura FF-AM, el Rhod 2-AM y el ácido plurónico se obtuvieron de Invitrogen-Molecular Probes (Carlsbad, CA).

-El Trizol™ y el Syber Green se adquirieron a Life Technologies.

-La ω-agatoxina-IVA se obtuvo de Peptide Institute (Sandhausen, Germany).

-La ω-conotoxina GVIA se obtuvo de Bachem Feinchemikalien (Budendorf, Switzerland).

-Todos los demás compuestos químicos utilizados habitualmente en el laboratorio se obtuvieron de Merck y/o Panreac Química (Madrid, Spain).

Las toxinas ω-agatoxina-IVA y ω-conotoxin GVIA se disolvieron en agua destilada a una concentración de 0.1 mM; el nifedipino se preparó en dimetilsulfóxido (DMSO) a una concentración de 0.01 mM y protegido de la luz. Las concentraciones finales (soluciones experimentales) de todos los fármacos se obtuvieron disolviendo las distintas soluciones stock directamente en las soluciones experimentales a emplear. A estas diluciones los solventes utilizados (DMSO, principalmente) no producen ningún efecto significativo sobre los distintos parámetros estudiados.

3.8.-TRATAMIENTO ESTADÍSTICO DE LOS DATOS

Los datos presentados en esta Tesis corresponden a las medias $\pm$ error estándar del número de datos habitualmente expresado entre paréntesis en las gráficas correspondientes.

Las diferencias entre tratamientos se consideraron significativas cuando se alcanzó una $P < 0,05$. Las comparaciones entre grupos se hicieron mediante la prueba estadística de la t de Student, comparando entre las dos poblaciones de células control y SHR. También realizamos pruebas de ANOVA para comparar las respuestas a tres tratamientos diferentes (CRT, FCCP, CRT+FCCP) comparando igualmente las dos poblaciones de células control y SHR.

RESULTADOS

4.-RESULTADOS

Se incluyen a continuación los artículos originales publicados en revistas SCI que constituyen la parte experimental de la presente Tesis Doctoral.

1. Quantal catecholamine release from adrenal chromaffin cells of control and hypertensive rats.
2. Role of the endoplasmic reticulum and mitocondria on quantal catecholamine release from chromaffin cells of control and hypertensive rats.
3. Greater cytosolic and mitocondrial calcium transients in adrenal medullary slices of hypertensive, compared with normotensive rats.
4. Lower density of L-type and higher density of P/Q-type of calcium channels in chromaffin cells of hypertensive, compared with normotensive rats.

FERREIRA, R.M., **DE PASCUAL, R.**, DE DIEGO, A.M.G., CARICATI-NETO, A., GANDIA, L., JURKIEWICZ, A. and GARCIA, A.G.

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Single-Vesicle Catecholamine Release Has Greater Quantal Content and Faster Kinetics in Chromaffin Cells from Hypertensive, as Compared with Normotensive, Rats

Regiane Miranda-Ferreira, Ricardo de Pascual, Antonio M. G. de Diego, Afonso Caricati-Neto, Luis Gandía, Aron Jurkiewicz, and Antonio G. García

Instituto Teófilo Hernando para la Investigación de Fármacos y del Envejecimiento, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain (R.M.-F., R.d.P., A.M.G.d.D., L.G., A.G.G.); Departamento de Farmacología, Escola Paulista de Medicina, Universidade Federal de Sao Paulo, Sao Paulo, Brazil (R.M.-F., A.C.-N., A.J.); and Servicio de Farmacología Clínica, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain (A.G.G.)

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ABSTRACT

In a previous study performed in the intact adrenal gland (Lim et al., 2002), stimulation with acetylcholine (ACh) or high K^+ concentrations (K^+) produced greater catecholamine release in spontaneously hypertensive rats (SHR), as compared with normotensive animals. In this study, the time course of secretion was in the range of minutes. Hence, we do not know whether enhanced release is due to greater quantal content and/or distinct kinetics in SHRs and control animals. To get insight into the mechanism involved in such enhanced catecholamine secretory responses, we performed a single-vesicle release study in primary cultures of adrenal chromaffin cells, recorded with amperometry. Cells were stimulated with 2-s pulses of 1 mM ACh or 70 mM K^+. The secretory responses to ACh or K^+ pulses in SHR cells as compared with control cells had the following characteristics: 1) double number of secretory events, 2) 4-fold augmentation of total secretion, 3) cumulative secretion that saturated slowly, 4) 3-fold higher complex events with two to four superimposed spikes that may be explained by faster spike kinetics, 5) about 2- to 3-fold higher event frequency at earlier post stimulation periods, and 6) 2- to 5-fold higher quantal content of simple spikes. We conclude that SHR cells have faster and larger catecholamine release responses, explained by more vesicles ready to undergo exocytosis and greater quantal content of vesicles. This could have relevance to further understand the pathogenic mechanisms involved in the development of high blood pressure, as well as in the identification of new drug targets to treat hypertension.

The differential release of the catecholamines norepinephrine and epinephrine from the adrenal medullary gland into the circulation, either in basal or stressful conditions, is tightly regulated by central (Folkow and Von Euler, 1954) and various peripheral splanchnic nerve stimulation patterns (Mirkin, 1961; Klevans and Gebber, 1970). Alteration of the activity of the sympathoadrenal medullary axis and of the rate of catecholamine release has been implicated in the pathogenesis of genetic essential hypertension, as proven by the classical use of drugs interfering with this axis to treat hypertensive patients i.e., blockers of α and β adrenergic receptors, reserpine, α-methyldopa, ganglionic blocking agents, guanethidine, angiotensin II receptor blockers, and so on (Westfall and Westfall, 2007). This is supported by the fact that the plasma levels of norepinephrine and epinephrine are augmented in spontaneously hypertensive rats (SHR) (Iriuchijima, 1973; Grobecker et al., 1975; Pak, 1981), as happens to be the case in humans suffering essential hypertension (Goldstein, 1983). This may be due to elevated sympathetic nerve activity, as revealed in hypertensive patients using microneurography (Anderson et al., 1989).

Numerous studies performed with different methodologies

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ABBREVIATIONS: SHR, spontaneously hypertensive rat; ACh, acetylcholine; DMEM, Dulbecco's modified Eagle's medium; ANOVA, analysis of variance; pC, picoculomb(s).

support that pre- and postsynaptic sympathetic dysfunctions are involved in the pathophysiology of primary hypertension in humans or laboratory animals (Tsuda and Masuyama, 1991; de Champlain et al., 1999). Studies on presynaptic mechanisms have been performed in the SHR, a model of primary hypertension; they show that norepinephrine release is increased in different tissues rich in sympathetic nerve endings (Donohue et al., 1988). Although this increase of NA release from sympathetic nerve endings constitutes an important catecholaminergic dysfunction associated to primary hypertension, the precise mechanism involved in this dysfunction remains unknown.

A recent study on catecholamine release from intact perfused rat adrenal glands indicates that release stimulated by acetylcholine (ACh), the physiological neurotransmitter at the adrenal medulla splanchnic nerve-chromaffin cell synapse (Feldberg et al., 1934), or by high K^+ concentrations (K^+), is higher in adrenals from SHR, as compared with the responses obtained in control normotensive rats (Lim et al., 2002). However, in this study, secretion was assessed upon collection of 4-min perfusate samples, with a poor temporal resolution to study a fast secretory event such as exocytosis of chromaffin cells (Neher, 1998). This low-temporal resolution precluded the analysis in such study of the fast kinetics of secretion or quantal aspects of single-vesicle secretory events upon stimulation of isolated individual chromaffin cells from control and SHR animals.

Thus, we felt that a study to analyze the kinetics of single-vesicle secretory events, using a carbon fiber electrode and amperometry, was timely. This high-resolution technique provides insight not only on quantitative, but also on qualitative, kinetic aspects of the last fusion steps of exocytosis in isolated chromaffin cells in the millisecond time range (Wightman et al., 1991; Borges et al., 2005). We found that short pulses (2 s) of ACh or K^+ elicited a more sustained production of spike secretory events and a drastic augmentation of the quantal catecholamine content of individual secretory vesicles, which had faster fusion kinetics in SHR, as compared with normotensive rats.

Materials and Methods

Control and Spontaneously Hypertensive Rats. Animals were manipulated according to the guidance of the Ethics Committee for Handling Research Animals of our medical school, Universidad Autónoma de Madrid. Male 16-week-old Sprague-Dawley and SHR weighing around 300 g were housed at 24 ± 2°C with 60 ± 20% relative humidity on a 12-h light/12-h dark cycle. Animals were fed a standard diet and water ad libitum and periodically weighed. Systolic blood pressure, diastolic blood pressure, and heart rate (beats per minute) were evaluated with a pressure meter (LETICA 2006; Cibertec, Madrid, Spain) by cannulating the femoral artery. As in our present study, several other studies on hypertensive rats used Sprague-Dawley rats as control normotensive rats (Wexler, 1980; Chao and Chao, 1988; Jandeleit-Dahm et al., 1997; Shimosawa et al., 2004).

Isolation and Culture of Rat Adrenal Medulla Chromaffin Cells from Control and Hypertensive Rats. To prepare each cell batch, we used one to two adult rats that were killed by cervical dislocation. The abdomen was opened, the adrenal glands were exposed and quickly removed and decapsulated, and both adrenal medullae were isolated under a stereoscope. They were placed in Ca^{2+}- and Mg^{2+}-free Locke buffer of the following composition: 154 mM NaCl, 3.6 mM KCl, 5.6 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM Hepes, pH 7.2, at room temperature. Tissues were collected under sterile conditions. Medullae digestion was achieved by incubating the pieces in 6 ml of Ca^{2+}/Mg^{2+}-free Locke buffer containing 6 mg of collagenase and 12 mg of bovine serum albumin for 20 min at 37°C; gentle agitation was applied at 5- to 10-min intervals by using a plastic Pasteur pipette. The collagenase was washed out of the cells with large volumes of Ca^{2+}/Mg^{2+}-free Locke buffer. The cell suspension was centrifuged at 120*g* for 10 min. After washing two times, the cells were resuspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum containing 50 IU/ml penicillin and 50 μg/ml streptomycin.

Cells were plated on circular glass coverslips, previously treated with 0.1 mg/ml poly-D-lysine for 30 min, followed by a thorough washout with water. After 30 min, 1 ml of DMEM was added to each well. Cells were then incubated at 37°C in a water saturated, 5% CO_2 atmosphere; they were used within 1 to 2 days after plating.

Amperometric Monitoring of Catecholamine Release with a Carbon Fiber Microelectrode. Carbon fiber microelectrodes were prepared by cannulating a 7-μm-diameter carbon fiber in polyethylene tubing. The carbon fiber tip was glued into a glass capillary for mounting on a patch-clamp head stage and backfilled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. The electrode was positioned at the middle right side of a spherical cell, gently touching the cell. Amperometric currents were recorded using an EPC-9 amplifier and PULSE software running on an Apple Macintosh computer (Apple Computer, Cupertino, CA). Sampling was performed at 14.5 kHz, and samples were digitally filtered at 2 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments, using 50 μM epinephrine as standard solution. Only fibers that rendered 200 to 300 pA of current increment after a 50 μM epinephrine pulse were used for the experiments. Cell secretion was stimulated by pulses of 70 mM K^+ or 1 mM ACh for 2 s, delivered from a micropipette located 40 μm away from the cell right side of the cell being explored; solutions bathed the cells by gravity, upon opening of computer-driven valves.

Spike Analysis and Statistics. Spike analysis was performed using the Pulse Program (HEKA, Lambrecht/Pfalz, Germany) and Igor Pro Software (Max Planck Institute, München, Germany), which includes the Ricardo Borges's macro package that allows the analysis of single events (Segura et al., 2000). A threshold of 4.5 times the first derivative of the noise S.D. was calculated to clearly detect amperometric events.

Differences between means of group data fitting a normal distribution were assessed by using Student's *t* test. A *p* value equal or smaller than 0.05 was taken as the limit of significance. The cumulative secretion (Figs. 1C and 2C) was analyzed by repeated measures with ANOVA followed by the Bonferroni test.

Materials and Solutions. The following materials were used: collagenase type I and ACh from Sigma-Aldrich (Madrid, Spain); and DMEM, bovine serum albumin fraction V, fetal calf serum, and antibiotics were from Invitrogen (Madrid, Spain). All other chemicals used were reagent grade from Merck and Panreac Química (Madrid, Spain).

Results

Body Weight, Adrenal Weight, and Hemodynamic Parameters of Control and SHR. Sixteen-week-old rats were used. Control rats weighed 328.2 g, and SHR weighed 318.9 g (Table 1). The dry weights of adrenal glands were 22.3 mg for control and 22.7 mg for SHR. The hemodynamic parameters were significantly higher in SHR as shown in Table 1. Thus, the systolic blood pressure, the diastolic blood pressure, and the mean arterial pressure were 55.2, 59.3, and 56.4% higher than the values obtained in control animals. The basal heart rate of control rats was 337 bpm and that of SHR animals 371 bpm, 10.2% higher.

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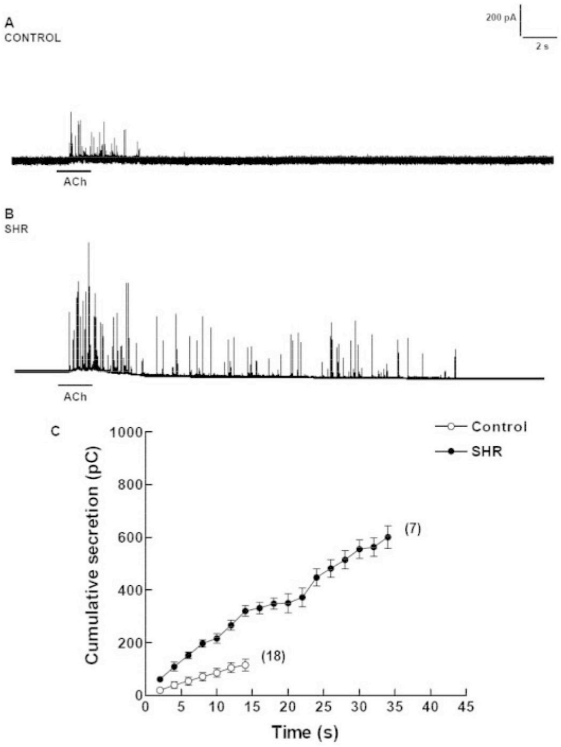

Fig. 1. ACh pulses produced longer lasting catecholamine secretory responses in SHR chromaffin cells, compared with control cells. A, example record obtained from a control cell stimulated with an ACh pulse (1 mM ACh, 2 s), as indicated by the horizontal bar at the bottom. B, record obtained from an example SHR cell, similarly stimulated with an ACh pulse. Note the prolonged duration of the period showing poststimulus amperometric spikes. C, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the area of spikes is expressed and represented in pC (ordinate) as a function of time after the ACh pulse (abscissa). Data are means ± S.E. of the number of cells shown in parentheses. Statistical difference between control and SHR cells was at $p < 0.001$ (***), using Bonferroni test after analysis by repeated measures with ANOVA.

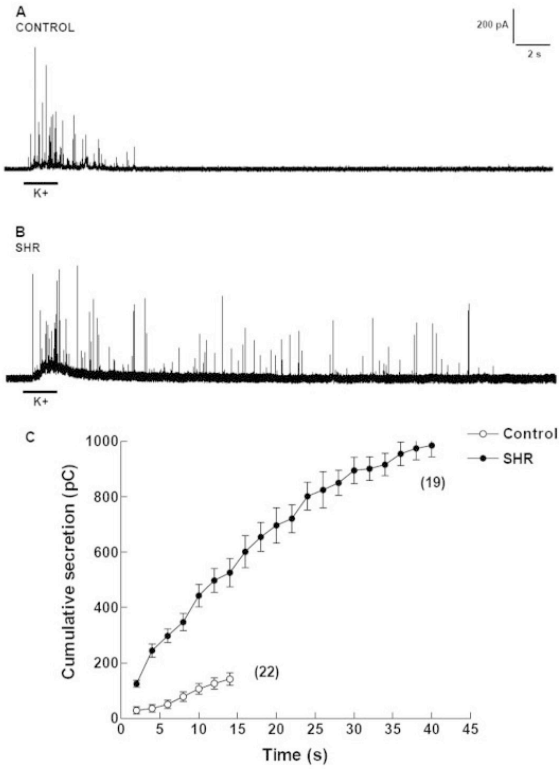

Fig. 2. High-K^+ pulses produced longer lasting catecholamine secretory responses in SHR chromaffin cells, as compared with control cells. A, example record obtained from a control cell stimulated with a high-K^+ pulse (70 mM K^+, 2 s), as indicated by the horizontal bar at the bottom. B, record obtained from an example SHR cell, similarly stimulated with K^+. Note the striking poststimulus secretory spike activity in this SHR cell. C, cumulative secretion, calculated at 2-s intervals from traces similar to those of A and B and represented in pC (ordinate) as a function of time. Data are means ± S.E. ***, $p < 0.001$, ANOVA.

The Amperometric Catecholamine Secretory Responses Elicited by ACh. Occasionally, rat adrenal chromaffin cells maintained in primary cultures fire low-frequency spontaneous action potentials (Kidokoro et al., 1982). This predicts that cells might spontaneously produce some secretory spikes. However, in our experiments, this spontaneous activity was rare, and when produced, the cell was discarded.

Usually, an experiment began with about 2- to 5 min of cell perifusion with the basal Krebs-Hepes solution to allow its equilibration. When possible, two or more cells were explored with the carbon fiber microelectrode in the same coverslip, going from right (the position of the microelectrode) to left (the position of the local superfusion pipette). In this manner, we did not expose to ACh or K^+ stimuli the second or third cell explored in the same coverslip.

Figure 1A shows a prototype secretory response elicited by an ACh pulse (1 mM, given focally for 2 s) in a control cell. After a delay of about 600 ms, a burst of secretory spikes appeared. The duration of the burst overlasted the ACh application by about 2.5 s. The spike frequency was initially higher to decline after 2 s or so. Note that the rest of the trace was silent, and no spikes clearly different from noise were produced.

The behavior of the SHR cell shown in the trace of Fig. 1B was quite different. First, the delay between the ACh pulse and the appearance of the first spike was considerably shortened to about 150 ms. Second, the baseline secretion was markedly enhanced likely as a result of overlapping secretory spikes. Third, the amplitudes of some spikes seemed to be larger. Fourth, the spike response overlasted the ACh pulse duration during a substantially longer period, about 15 s.

Figure 1C shows a plot of the mean cumulative secretion from various cells explored following the protocol of Fig. 1, A and B. The secretion evoked by the ACh pulse in each individual cell was calculated at 2-s intervals because the area of spikes present within such 2-s period that was expressed in picoculombs (pC) of catecholamine released and cumulatively added to obtain the curves of Fig. 1C. Note that in control cells, secretion during the first 6 s after the ACh pulse saturated at about 120 pC and stopped after 14 s. In contrast, in

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TABLE 1
Body weight, adrenal gland weight, and hemodynamic parameters of control and SHR rats
Data are means ± S.E. of the number of animals shown in *n*. In parentheses, the percent increases above control rats are given.

	n	Body Weight	Adrenal Gland Weight	SBP	DBP	MAP	Heart Rate
		g	*mg*		*mm Hg*		*bpm*
Control	20	328.2 ± 2.67	22.3 ± 0.51	125.7 ± 0.63	99.3 ± 0.68	109.1 ± 0.74	336.7 ± 1.31
SHR	20	318.9 ± 1.98	22.75 ± 0.53	195.1 ± 1.2* (155%)	158.2 ± 1.7* (159%)	170.6 ± 1.5* (156%)	371 ± 2.6* (110%)

n, number of animals; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean blood pressure; bpm, beats per minute.
* $p < 0.001$ (Student's *t* test).

SHR cell secretion continued rising to about a 600 pC peak at the 35th s.

The Amperometric Catecholamine Secretory Responses Elicited by K^+. Saline solutions containing high K^+ concentrations cause chromaffin cell depolarization, enhanced Ca^{2+} entry, and catecholamine release. This stimulus directly recruits voltage-dependent Ca^{2+} channels, thus bypassing the nicotinic receptors stimulated by ACh (Garcia et al., 2006). Hence, to define whether ACh evoked greater responses in SHR cells just through a nAChR-delimited effect, secretion was also studied in cells stimulated with K^+-depolarizing saline solutions.

In the experiments shown in Fig. 2A, a protocol similar to that of Fig. 1 was followed, except for the fact that here, a control cell was stimulated for 2 s with a solution containing 70 mM K^+ (isoosmolar reduction of Na^+). Note that the secretory spikes appeared soon after K^+ application, with a delay of only 200 ms; the secretory activity overlasted the K^+ pulse and kept going for an additional 4-s period.

The trace of Fig. 2B was obtained from an SHR cell. Here, a pronounced elevation of baseline secretion, indicating the superposition of many catecholamine release quanta, was seen. Furthermore, the secretory response continued for about 20 to 30 s after the K^+ pulse. Figure 2C shows that in control cells, cumulative secretion gradually rose to reach a plateau at the 10th s, at about 150 pC; the secretory activity stopped after 10 s of the K^+ pulse. In contrast, secretion in SHR cells lasted much longer and rose to a plateau of near 1000 pC after 40 s of the K^+ pulse.

Decay of Secretory Responses upon Repeated Pulsing with ACh or K^+. In a few SHRs and in most control cells, repeated pulsing with ACh or K^+ produced secretory responses that decayed with time. For instance, in four SHR cells, we could obtain two responses evoked by ACh pulses

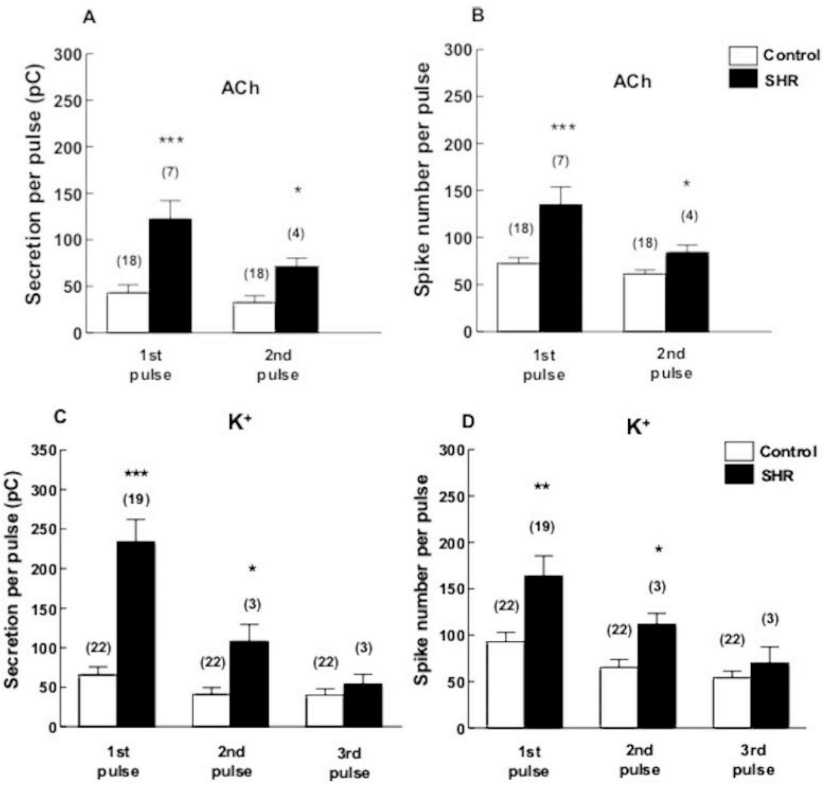

Fig. 3. Decay of secretory responses upon repeated cell pulsing with ACh (1 mM, 2 s) or K^+ (70 mM, 2 s) is much more pronounced in SHR cells, as compared with control cells. A, total secretion per pulse (integrated area of all spikes obtained in each stimulus, in pC, ordinate) in control and SHR cells stimulated twice with a 5-min interval. B, total number of spikes secreted per each ACh pulse in the same cells of A. C and D, total secretion and spike number per pulse, respectively, in cells stimulated with three successive K^+ pulses given at 5-min intervals. Data are means ± S.E. of the number of cells given in parentheses. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, with respect to control cells (Student's *t* test).

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separated by a 5-min interval. Figure 3A shows that the second secretory responses decayed to about 50% in control cells and by 40% in SHR cells. However, the responses to both stimuli were significantly higher in SHR cells, about 2.5-fold above control cells. The number of spikes also decreased during the second ACh pulse in both cell types, but once more, it was higher in SHR cells.

Secretory responses also decayed with successive high-K^+ pulses (Fig. 3C). For instance, in three SHR cells, the total secretion per pulse (Q) decreased from 220 pC (first pulse) to about 100 pC (second pulse) and to around 60 pC (third pulse). A similar relative decline was seen in control cells, although such decline was smaller from the second to the third K^+ stimulus. Again, secretion was 3- to 4-fold higher in SHR cells, as compared with control cells during the first and second stimuli. A similar picture was observed on the total number of spikes per K^+ pulse; spike numbers were higher in SHR cells during the first and second K^+ stimuli, whereas they were similar to control cells during the third stimulus.

Since the decay of secretion to repeated pulsing was high, particularly in SHR cells, we performed the kinetic analysis of single-vesicle events by taking into account only the secretory responses elicited by each ACh or K^+ pulse in every individual cell.

Incidence of Single and Multiple Spikes in the Secretory Responses Elicited by ACH or K^+. To analyze some kinetic aspects of individual secretory spikes, we investigated first the incidence of single separated spikes and that of secretory complexes consisting of multiple spikes that did not reach baseline secretion. Figure 4 shows four examples of these spike types. It is known that in about 10% of the cases, partial vesicle fusion proceeds to a full fusion event, resulting in a foot signal preceding an amperometric spike (Zhou et al., 1996). Most of the spikes obtained in this study had no foot, as the prototype shown in Fig. 4A. The spike shown in Fig. 4B has a foot that proceeds to a full fusion spike. Figure 4C shows a secretory event consisting of two partially superim-

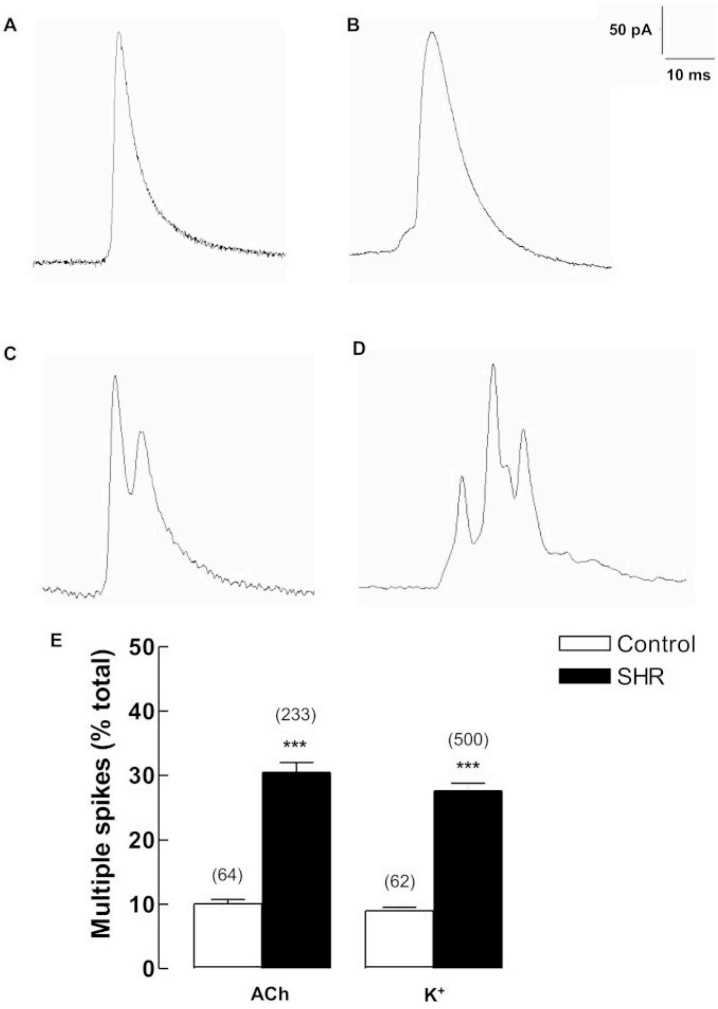

Fig. 4. Incidence of single and multiple amperometric spike events in the secretory responses evoked by ACh (1 mM, 2 s) or high K^+ pulses (70 mM K^+, 2 s), in control and SHR cells. A and B, examples of single spikes, with or without a foot. C and D, samples of multiple spikes. To draw the histogram of E, single or multiple spikes were counted in the trace secretory responses elicited by ACh or K^+. The incidence of multiple spikes in each individual cell was expressed as percentage of total spikes, counting single or "multiple" spikes as "individual" events. Data are means ± S.E. ***, $p < 0.001$ with respect to control (Student's t test).

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posed spikes, and Fig. 4D shows a complex secretory event with four spikes.

We counted the number of single or multiple spikes in all traces recorded from control and SHR cells stimulated with ACh or K^+ pulses (Fig. 4E). In control cells stimulated with ACh, 10% of the 599 spikes analyzed had a complex profile. In SHR cells, 30% of spikes were complex, of the 834 spikes analyzed. A similar picture emerged upon the analysis of K^+-elicited spikes; multiple spikes accounted for 9% in control cells and were 3.5-fold more frequent in SHR cells.

Frequency Distribution of Secretory Events in the Responses Elicited by ACh or K^+ Pulses. Another facet of interest was the analysis of spike frequency along the traces obtained in the example cells shown in Figs. 1 and 2. Such analysis was performed second by second and is presented in Fig. 5A, showing the responses elicited by ACh. During the first 9 s of the trace, the spike frequency was in general higher in SHR, compared with control cells. From this time to the 27th s, the control cell was silent, whereas the SHR cell continued discharging spike events at about 5 to 7 Hz.

The responses to K^+ were considerably faster during the first 10 s, as indicated in Fig. 5B. For instance, during the first 5 s, the control spikes reached frequencies between 7 to 17 Hz (compared with about 10 Hz with ACh); the spikes from SHR cells reached frequencies as high as 40 Hz.

Frequency Distribution of Secretory Events as a Function of Spike Amplitude. A significant higher spike number was counted along the secretory responses elicited by ACh or K^+ in SHR cells, with respect to control cells. The question is whether the distribution of spikes was uniform at different spike amplitudes, or rather, if they were randomly distributed along the trace recording obtained with each stimulus, independently of amplitudes.

Figure 6A shows the number of spikes generated by ACh pulsing as a function of amplitude ranges, grouped in 200-pA steps. In the lower amplitude range (0–199 pA), 39 spikes were found in control cells and 76 spikes in SHR cells, about

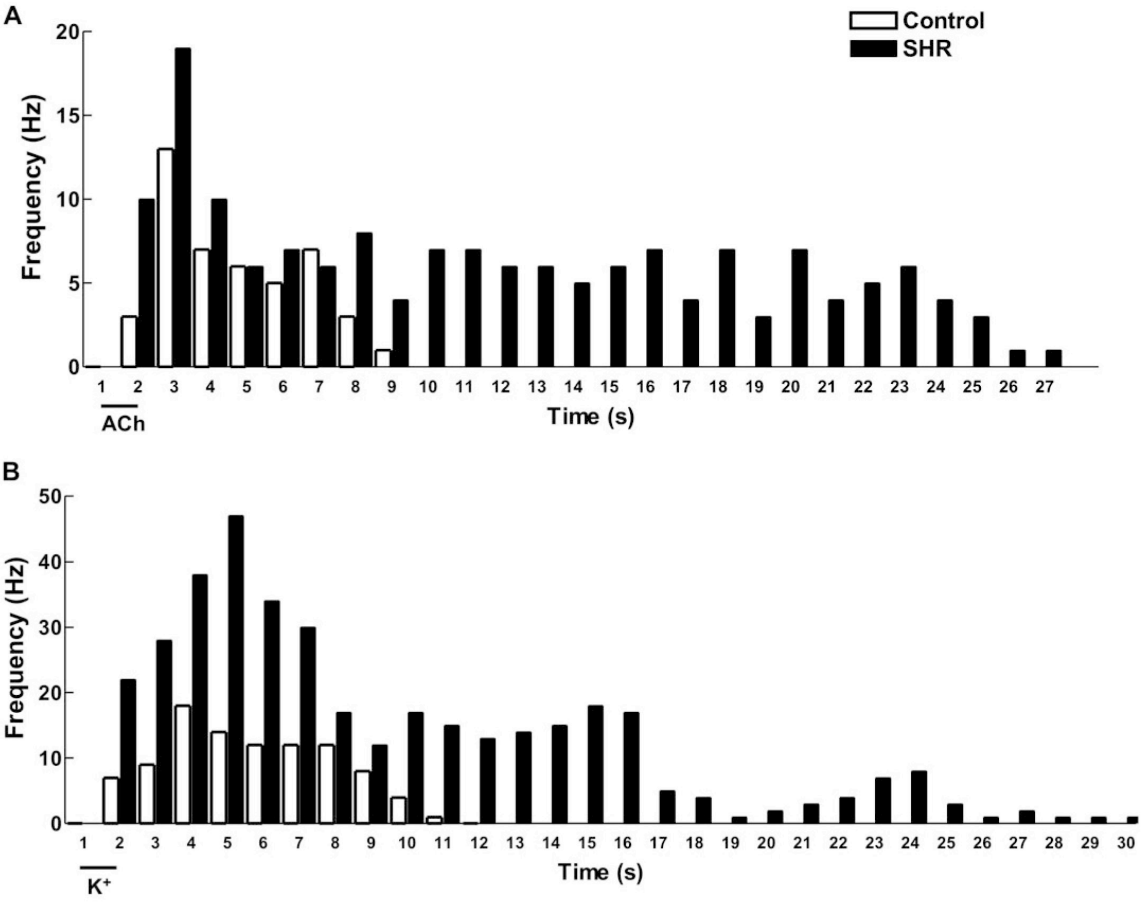

Fig. 5. Histograms of frequency spike distribution, calculated from the traces shown in Figs. 1, A and B, and 2, A and B, corresponding to the secretory responses elicited by ACh or K^+, in control and SHR cells. The number of spikes present in 1-s time periods were calculated and plotted as frequencies in Hz (ordinates). Data are representative of cells stimulated with a 2-s ACh pulse (as indicated by the bottom horizontal bar in A) or by a 2-s K^+ pulse (as shown by the horizontal bar in B).

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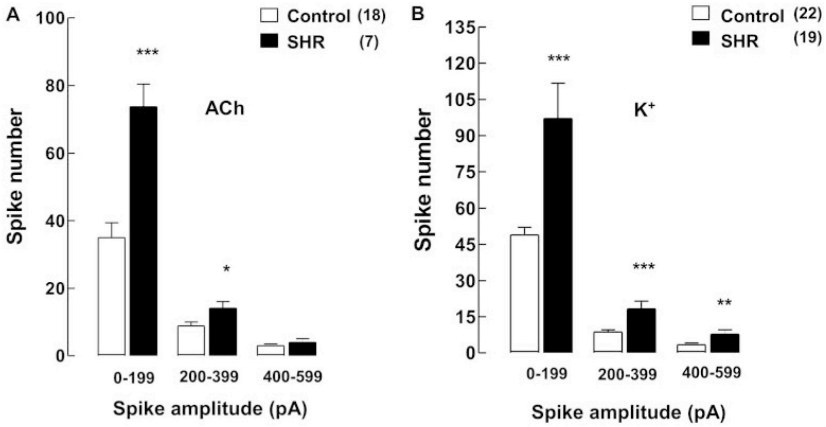

Fig. 6. Distribution of secretory spikes as a function of their amplitudes, in control and SHR cells stimulated with ACh or K^+ pulses. Spikes from all traces obtained in the experiments shown in Figs. 1 and 2 were grouped according to their amplitudes in the 200-pA range (abscissae), counted, and their numbers are given in the ordinates. A, spike distribution in cells stimulated with ACh pulses; B, that obtained from cells stimulated with K^+ pulses. Data are means ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, with respect to control cells (Student's t test).

2-fold higher. In the range of 200 to 399 pA, these values decreased to 9 and 14 spikes, respectively. The spikes with higher amplitudes were few and had no statistical differences between control and SHR.

In the case of spikes elicited by K^+, a similar pattern emerged. So, in the lower range (0–199 pA), control cells exhibited 54 spikes, whereas SHR cells had 103 spikes, about 2-fold higher. In the range of 200 to 399 pA, nine spikes were in control cells and 19 spikes in SHR cells. Again, at higher amplitudes, fewer spikes were seen.

Catecholamine Quantal Content and Kinetics of Single Amperometric Spikes. We also analyzed the quantal catecholamine content of individual spikes (Q, in pC). Partially superimposed spikes that begun above baseline or that did not reach baseline when decaying were hard to accurately estimate their area and hence their quantal catecholamine contents; therefore, we did not include them in this analysis. The area of these single spikes reflects the release of the total vesicular content of catecholamines (Zhou et al., 1996).

Spikes were distributed in groups according to their amplitudes, in 200-pA steps. The maximal numbers of spikes were found in the range of 0- to 199-pA amplitude, both for ACh and K^+ pulses, in control as well as SHR cells (Fig. 7, A and B). In this range, the quantal content of spikes from control cells stimulated with ACh approached 1 pC, in agreement with Q values of 0.8 pC of mouse chromaffin cells (Arroyo et al., 2006) and 1.3 pC in bovine chromaffin cells (Ardiles et al., 2007). A surprising finding was the much higher Q of SHR spikes, about 3- to 5-fold higher than the values of Q for control cells stimulated with ACh. The spikes triggered by K^+ exhibited a pattern similar to ACh (Fig. 7B); their quantal content was about 2- to 4-fold higher in SHRs, compared with control cells.

We also analyzed the kinetics of individual spikes. Table 2 shows that the time to peak (t_{max}) was around 16 ms in spike events elicited by ACh or K^+ stimulation of control cells; t_{max} was reduced by about 40% in SHR spikes. This indicated faster spike ascension (m) that was 35 to 50% higher in SHR cells, compared with control cells. The spike half-width ($t_{1/2}$) was reduced by 21% in SHR cells.

Discussion

We have found that the stimulation of single rat adrenal chromaffin cells with short ACh or K^+ pulses causes catecholamine secretory responses that are substantially higher and longer lasting in cells obtained from SHRs, as compared with responses generated in cells from control normotensive animals (Figs. 1–3). This corroborates the observation of Lim et al. (2002), who in the perfused intact adrenal gland observed that stimulation with ACh or K^+ enhanced more the catecholamine present in 4-min perfusate samples of SHRs, as compared with control rats. However, in such a study using the intact gland, the slow perfusion rate and the low

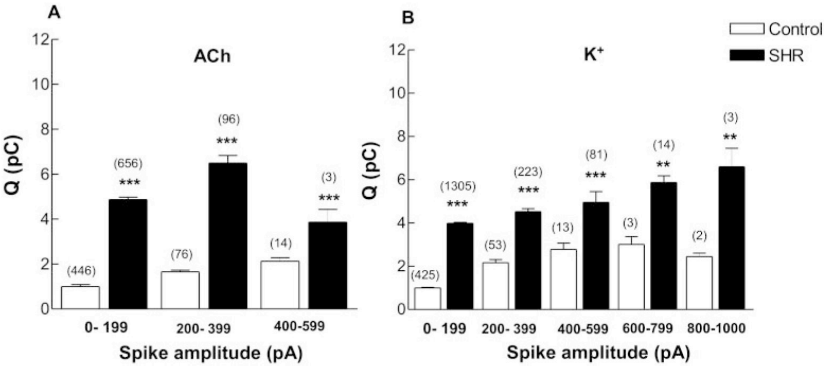

Fig. 7. Analysis of the quantal content of individual secretory events obtained from control and SHR cells (Figs. 1 and 2). Spike areas (quantal catecholamine content of individual vesicles, expressed in pC, Q, in the ordinates) were individually analyzed and distributed according to the amplitude range, in pA, shown in the abscissae. Data are means ± S.E. of the number of spikes shown in parentheses on top of each column. A, analysis of spikes obtained with ACh stimulation of control and SHR cells. B, corresponding to K^+ stimulation. Data are means ± S.E. of the number of spikes shown in parentheses on top of each column. **, $p < 0.01$; ***, $p < 0.001$, with respect to control cells.

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TABLE 2
Kinetic parameters of the spike secretory events in control and SHR cells

	t_{max} (ms)	m (pA/ms)	$t_{1/2}$ (ms)	n
Control				
ACh	16.1 ± 0.83	31.9 ± 3.0	9.8 ± 0.2	584
K^+	15.7 ± 2.2	55.3 ± 3.9	7.6 ± 0.2	678
SHR				
ACh	9.4 ± 1.8*** (58.4%)	43.3 ± 3.2* (135.7%)	7.7 ± 0.3*** (78.5%)	712
K^+	10.4 ± 0.8* (66.2%)	84.9 ± 3.2*** (153.5%)	5.9 ± 0.1*** (77.6%)	1638

Data are means ± S.E. of the number spikes (n). m, rate of spike ascension; $t_{1/2}$, spike half-width.
* $p < 0.01$, compared with their respective ACh or K^+ controls (Student's t test).
*** $p < 0.0001$, compared with their respective ACh or K^+ controls (Student's t test).

sensitivity of the catecholamine fluorescence assay permitted only the analysis of global catecholamine release responses in the minute range, precluding to get insight into the kinetic mechanisms involved in such enhanced secretion. In our study, we measured the amperometric detection of single-vesicle exocytotic events in cultured single chromaffin cells, allowing us a high temporal resolution (milliseconds to seconds) analysis of secretory responses. We found that the integrated total catecholamine release elicited by an ACh pulse was 4-fold higher in SHRs, as compared with control rats (Fig. 1C). However, the number of secretion spikes generated by an ACh pulse was only 2-fold higher in SHR cells (Fig. 1D). In cells stimulated with K^+ pulses, a similar outcome was seen (Fig. 2, C and D). Thus, augmented secretion in SHRs versus control cells may have at least two components: 1) a higher number of secretory events and 2) a higher quantal content of individual events, as shown in Fig. 7.

Another interesting feature was related to the number of events having single or multiple spikes. It is generally accepted that a separate well identified spike corresponds to the quantal total release of the catecholamine stored in a single secretory vesicle, through a fusion pore formed by the fusion of the vesicle membrane with the plasmalemma (Breckenridge and Almers, 1987); this pore usually expands to release the total vesicular content expressed by the area of a single spike (Zhou et al., 1996). Multiple spike events indicate that various vesicles suffered almost simultaneous exocytosis; this was more pronounced with K^+ stimulation of SHR cells, which caused a clear elevation of baseline secretion (Fig. 2B). In SHR cells, about 30% of events had multiple spikes (two to four spikes), whereas control cells had only 10% of events with multiple spikes; this was true with ACh or K^+ stimulation (Fig. 4E), suggesting that in SHR cells, more vesicles were docked and primed at subplasmalemmal sites (Neher, 1998) to undergo exocytosis upon ACh or K^+ stimulation. Alternatively, it may also indicate that vesicle fusion with the plasmalemma and the ensuing quantal catecholamine release was faster (Table 2), giving rise to more exocytotic sites available and to more spike overlapping.

It is unlikely that differences in the nicotinic receptors and/or voltage-dependent Ca^{2+} channels present in rat chromaffin cells (Garcia et al., 2006) could explain the different secretion pattern seen in SHRs, as compared with control cells. This conclusion is supported by the quite similar responses generated by ACh, which indirectly depolarizes the chromaffin cells (Douglas et al., 1967) and activates exocytosis by enhancing Ca^{2+} entry through Ca^{2+} channels (Douglas and Poisner, 1961) or by high K^+ concentrations that cause direct cell depolarization (Douglas et al., 1967) and recruitment of voltage-dependent Ca^{2+} channels in both control and SHR cells. However, it will be interesting to study the inward currents through nicotinic receptors generated by ACh, as well as the inward Ca^{2+} currents generated by square depolarizing pulses or by action potentials in both normotensive and hypertensive rats.

Another possibility rests in a different Ca^{2+} homeostatic mechanism in SHRs as compared with control cells. We have seen different patterns of exocytosis in bovine and mouse chromaffin cells, likely related to different Ca^{2+} handling by mitochondria, upon cell depolarization with K^+ or electrically evoked depolarizing pulses (Ales et al., 2005). Also, we found differences in ACh- or K^+-evoked secretory responses in bovine chromaffin cell populations when the endoplasmic reticulum Ca^{2+} movements were disturbed (Cuchillo-Ibanez et al., 2002). A functional triad controlling $[Ca^{2+}]_c$ and exocytotic signals has been described to be present in bovine chromaffin cells using endoplasmic reticulum- or mitochondria-targeted aequorins and measurements of inward Ca^{2+} channel currents (Alonso et al., 1999; Montero et al., 2000; Garcia et al., 2006). It will be interesting to test whether such functional triad is present in chromaffin cells of control normotensive rats and if it is altered at some points in hypertensive rats. Alterations of these Ca^{2+} homeostatic mechanisms occur in vascular smooth muscle cells. In fact, a large Ca^{2+} influx and a high $[Ca^{2+}]_c$ are reported even during rest in conduit arteries (e.g., the aorta and the carotid and femoral arteries) from SHRs as compared with normotensive rats (Jelicks and Gupta, 1990; Asano et al., 1996); this has also been found in small mesenteric arteries from SHRs, where the sarcoplasmic reticulum has a larger capacity for Ca^{2+} storage (Nomura and Asano, 2002).

In conclusion, we have shown that short ACh or K^+ pulses cause catecholamine secretory responses that drastically differ in chromaffin cells of normotensive and hypertensive rats. The much greater catecholamine release responses in SHRs are explained by the faster exocytosis of more vesicles with greater quantal catecholamine content, during a much longer secretory period. This drastic different behavior of the chromaffin cell secretory responses may contribute to the understanding of the pathogenic mechanism of hypertension development in humans and facilitate the identification of novel therapeutic targets to control high blood pressure.

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Address correspondence to: Dr. Antonio G. García, Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 4. 28029 Madrid, Spain. E-mail: agg@uam.es

FERREIRA, R.M., **DE PASCUAL, R.**, CARICATI-NETO, A., GANDIA, L., JURKIEWICZ, A. and GARCIA, A.G.

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Role of the Endoplasmic Reticulum and Mitochondria on Quantal Catecholamine Release from Chromaffin Cells of Control and Hypertensive Rats

Regiane Miranda-Ferreira, Ricardo de Pascual, Afonso Caricati-Neto, Luis Gandía, Aron Jurkiewicz, and Antonio G. García

Departamento de Farmacología, Escola Paulista de Medicina, Universidade Federal de São Paulo, Sao Paulo, Brazil (R.M.-F., A.C.-N., A.J.); Instituto Teófilo Hernando, Madrid, Spain (R.d.P., L.G., A.G.G.); Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain (R.d.P., L.G., A.G.G.); and Servicio de Farmacología Clínica, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, Madrid, Spain (A.G.G.)

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ABSTRACT

Here, we present the first study on the effects of compounds that interfere with calcium (Ca^{2+}) handling by the endoplasmic reticulum (ER) and mitochondria on amperometrically measured quantal catecholamine release from single adrenal chromaffin cells of control and spontaneously hypertensive rats (SHRs). Acetylcholine (ACh) or K^+ pulses triggered spike bursts of secretion by Ca^{2+} entry through Ca^{2+} channels. ER Ca^{2+} release triggered by a mixture of caffeine, ryanodine, and thapsigargin (CRT) or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (a mitochondrial protonophore) also caused bursts of secretory spikes. The spike bursts generated by ACh, K^+, CRT, and FCCP were 3 to 4 times longer in SHRs compared with control cells; furthermore, the individual spikes were faster and had 3-fold greater quantal size. In additional experiments, a 90-s treatment was made with CRT or FCCP to block Ca^{2+} handling by the ER and mitochondria. In these conditions, the integrated spike burst responses elicited by ACh and K^+ were potentiated 2- to 3-fold in control and SHR cells. This suggests that variations in Ca^{2+} entry and its subsequent redistribution into the ER and mitochondria are not responsible for the greater secretion seen in SHRs compared with control cells; rather, such differences seem to be due to greater quantal content of spike bursts and to greater quantal size of individual amperometric events.

The differential release into the circulation of the catecholamines norepinephrine and epinephrine from the adrenal medullary gland, either in basal or stressful conditions, is tightly regulated by various central (Folkow and Von Euler, 1954) and peripheral splanchnic nerve stimulation (Mirkin, 1961; Klevans and Gebber, 1970) patterns. Alteration of the activity of the sympathoadrenal medullary axis and of the rate of catecholamine release has been implicated in the pathogenesis of essential hypertension, as proven by three facts: 1) the classic effects of drugs interfering with this axis, used to treat hypertensive patients such as blockers of α- and β-adrenergic receptors, reserpine, α-methyldopa, ganglionic blocking agents, guanethidine, or angiotensin II receptor blockers (Westfall and Westfall, 2007); 2) plasma levels of norepinephrine and epinephrine are augmented in spontaneously hypertensive rats (SHRs) (Iriuchijima, 1973; Grobecker et al., 1975; Pak, 1981), as happens to be the case in humans suffering essential hypertension (Goldstein, 1983); and 3) hypertensive patients have elevated sympathetic nerve activity, as revealed with microneurography (Anderson et al., 1989).

Numerous studies support the notion that pre- and postsynaptic sympathetic dysfunctions are involved in the pathophysiology of human or animal primary hypertension (Tsuda and Masuyama, 1991; de Champlain et al., 1999). Studies on presynaptic mechanisms have been performed in SHRs, showing that norepinephrine release is increased in different tissues rich in sympathetic nerve endings (Donohue

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ABBREVIATIONS: SHR, spontaneously hypertensive rat; ACh, acetylcholine; CRT, mixture of caffeine, ryanodine, and thapsigargin; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DMEM, Dulbecco's modified Eagle's medium.

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et al., 1988). Although this increase of norepinephrine release constitutes an important catecholaminergic dysfunction associated with primary hypertension, the precise mechanism involved remains unknown.

A study on catecholamine secretion from intact perfused rat adrenal glands indicated that release stimulated by acetylcholine (ACh) or by potassium (K^+) was higher in adrenals from SHRs compared with control normotensive rats (Lim et al., 2002b). We recently performed the first study on the kinetics of single-vesicle secretory events, using a carbon fiber electrode and amperometry in isolated single chromaffin cells from control and SHRs (Miranda-Ferreira et al., 2008). This high-resolution technique provides insight on quantitative and qualitative kinetic aspects of the last fusion steps of exocytosis in isolated single chromaffin cells in the millisecond time range (Wightman et al., 1991; Borges et al., 2005). We found that SHR chromaffin cells, stimulated with short pulses of ACh or K^+, had a more sustained production of spike secretory events and a drastic augmentation of the quantal catecholamine content of individual secretory vesicles with faster fusion kinetics, compared with normotensive rats (Miranda-Ferreira et al., 2008).

Differences in the nicotinic receptors and/or voltage-dependent Ca^{2+} channels in chromaffin cells (Garcia et al., 2006) could explain the different secretion patterns seen in SHRs compared with control rats. Alterations of intracellular Ca^{2+} homeostatic mechanisms could also account for the differences. In fact, a large Ca^{2+} influx and a high cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) has been seen even during rest in conduit arteries i.e., aorta, carotid, and femoral arteries from SHRs compared with normotensive rats (Jelicks and Gupta, 1990; Asano et al., 1996); this also has been found in small mesenteric arteries from SHRs, where the sarcoplasmic reticulum has a larger capacity for Ca^{2+} storage (Nomura and Asano, 2002).

In trying to clarify the mechanisms involved in the kinetic differences of single-vesicle quantal release of catecholamine between SHR and normotensive rats found in our recent study (Miranda-Ferreira et al., 2008), we felt it of interest to study whether alterations of Ca^{2+} handling by the endoplasmic reticulum and mitochondria could explain those differences in the exocytotic responses. Here, we present such a study in which we have compared the kinetics of quantal catecholamine release elicited by the following stimuli applied to single chromaffin cells from normotensive and hypertensive rats: 1) pulses of ACh or K^+, to favor Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Douglas and Poisner, 1961); 2) acute application of a mixture of caffeine, ryanodine, and thapsigargin (CRT) to quickly release the Ca^{2+} stored in the ER (Cuchillo-Ibáñez et al., 2002); 3) acute application of the protonophore FCCP to cause release into the cytosol of Ca^{2+} accumulated into mitochondria during previous cell stimulations with ACh or K^+ pulses (Montero et al., 2000); and 4) ACh or K^+ pulses given after cell treatments with CRT or FCCP. We demonstrate that: 1) quantal secretory responses elicited by Ca^{2+} entry or intracellular Ca^{2+} release were much longer in SHRs compared with controls; 2) the responses to ACh and K^+ were markedly enhanced in cells previously treated with CRT or FCCP; 3) the individual single-vesicle secretory spikes were faster and had a greater quantal size in SHRs compared with control rats.

Materials and Methods

Animals. Animals were manipulated according to the guidance of the Ethics Committee for Handling Research Animals of the Medical School of the Universidad Autónoma de Madrid (Madrid, Spain). Male 16-week-old Sprague-Dawley and SHR rats weighing approximately 300 g were housed at 24 ± 2°C, with 60 ± 20% relative humidity, on a 12-h light/dark cycle. Animals were fed a standard diet and water ad libitum and periodically weighed.

Isolation and Culture of Rat Adrenal Medulla Chromaffin Cells. To prepare each cell batch, we used one to two adult rats that were killed by cervical dislocation. The abdomen was opened, and the adrenal glands were exposed, quickly removed and decapsulated, and both adrenal medullae were isolated under a stereoscope. They were placed in Ca^{2+}- and Mg^{2+}-free Locke buffer of the following composition: 154 mM NaCl, 3.6 mM KCl, 5.6 mM $NaHCO_3$, 5.6 mM glucose, and 10 mM HEPES, pH 7.2, at room temperature. Tissues were collected under sterile conditions. Medullae digestion was achieved by incubating the pieces in 6 ml Ca^{2+}/Mg^{2+}-free Locke buffer containing 6 mg of collagenase and 12 mg of bovine serum albumin for 20 min at 37°C; gentle agitation was applied at 5- to 10-min intervals by using a plastic Pasteur pipette. The collagenase was washed out of the cells with large volumes of Ca^{2+}/Mg^{2+}-free Locke buffer. The cell suspension was centrifuged at 120*g* for 10 min. After washing two times, the cells were resuspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum containing 50 IU/ml penicillin and 50 μg/ml streptomycin.

Cells were plated on circular glass coverslips, previously treated with 0.1 mg/ml poly-D-lysine for 30 min, followed by a thorough washout with water. After 30 min, 1 ml of DMEM was added to each well. Cells were then incubated at 37°C in a water-saturated, 5% CO_2 atmosphere; they were used within 1 to 2 days after plating.

Amperometric Monitoring of Catecholamine Release with a Carbon Fiber Microelectrode. Carbon fiber microelectrodes were prepared by cannulating a 7-μm-diameter carbon fiber in polyethylene tubing. The carbon fiber tip was glued into a glass capillary for mounting on a patch-clamp head stage and back-filled with 3 M KCl to connect to the Ag/AgCl wire, which was kept at +700 mV. The electrode was positioned at the middle right side of a spherical cell, gently touching the cell. Amperometric currents were recorded using an EPC-9 amplifier and PULSE software running on an Apple Macintosh computer (Apple Computer, Cupertino, CA). Sampling was performed at 14.5 kHz, and samples were digitally filtered at 2 kHz. The sensitivity of the electrodes was routinely monitored before and after the experiments, using 50 μM epinephrine as standard solution. Only fibers that rendered 200 to 300 pA of current increment after a 50 μM epinephrine pulse were used for the experiments.

Experimental Protocols. Cell secretion was stimulated by pulses of 70 mM K^+ or 1 mM ACh for 2 s, delivered from a micropipette located 40 μm away from the right side of the cell being explored; solutions bathed the cells by gravity from left to right, upon opening of computer-driven valves. After the stimuli with ACh and/or K^+, cells were treated with a cocktail containing caffeine (20 mM), ryanodine (10 μM), and thapsigargin (1 μM) to study the role of endoplasmic reticulum Ca^{2+} on quantal catecholamine release. This cocktail will be referred to as CRT throughout. To study the role of mitochondria, cells were perifused with FCCP (1 μM). Both combined treatments were also used to study the participation of the two organelles on the secretory response. After 30 s with this treatment, the same cells were challenged with ACh or K^+ for 2 s, and then these cells were bathed with Tyrode's solution for 4 min to be challenged once more with ACh and K^+ pulses.

Spike Analysis and Statistics. Total cumulative secretion elicited by ACh and K^+ pulses or the transient initial secretion elicited by CRT, FCCP, or CRT/FCCP treatments were calculated by successive additions of individual spike areas (Q) and expressed in picoCoulombs. Baseline elevation during a given stimulus is due to spike overlapping caused by faster secretion (Miranda-Ferreira et

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al., 2008). Note that the number of spikes shown in the histograms in several figures may be underestimated because baseline elevation in some traces cannot be resolved in separate spikes. Thus, in some cases, a correlation between cumulative secretion and spike number elicited by a given treatment or stimulation was not obvious (see further comments under *Results*).

Kinetic analysis of individual spikes was performed using the Pulse Program (HEKA, Lambrecht/Pfalz, Germany) and Igor Pro Software (Max Planck Society, Munich, Germany), which includes the Ricardo Borges's macro package that allows the analysis of single events (Segura et al., 2000). A threshold of 4.5 times the first derivative of the noise S.D. was calculated to clearly detect amperometric events. We studied the integral charge, the individual charge, the number of spikes, and the kinetic parameters, i.e., time to peak (t_{max}), $t_{1/2}$, and amplitude (I_{max}) before and after the ER and mitochondrial treatments.

Differences between means of group data fitting a normal distribution were assessed by using Student's t test and analysis of variance (difference between treatments). A p value equal or smaller than 0.05 was taken as the limit of significance.

Materials and Solutions. The following materials were used: collagenase type I and ACh chloride and FCCP were from Sigma-Aldrich (St. Louis, MO), and DMEM, bovine serum albumin fraction V, fetal calf serum, antibiotics, caffeine, thapsigargin, and ryanodine were from Invitrogen (Carlsbad, CA). All other chemicals used were reagent grade from Merck and Panreac Química (Barcelona, Spain).

Results

The Quantal Catecholamine Release Responses Triggered by ACh or K^+ Pulses in Chromaffin Cells from Normotensive and Hypertensive Rats. All experiments began with an initial 5-min perifusion resting period to adapt the targeted cell to its environment. No spontaneous secretory amperometric spikes were usually seen during this period. Thus, the low-frequency spontaneous action potentials reported to occur in primary cultures of rat chromaffin cells (Kidokoro and Ritchie, 1980) were not apparently capable of eliciting exocytosis under our present experimental conditions.

To stimulate secretion, the basal Krebs-HEPES solution containing 2 mM Ca^{2+} was quickly switched to another containing 1 mM ACh that bathed the cell for 2 s (the duration of the ACh pulses). Figure 1A shows the spike burst produced by ACh in an example control cell. A given cell usually was stimulated five times with ACh at regular 4-min intervals; during those intervals, the cell remained silent. The responses to the second pulse decayed by 30% with respect to pulse 1; the following responses underwent little decay (data not shown). Thus, when used, pharmacological treatments (i.e., CRT of FCCP) were given between ACh pulses 2 and 3.

Figure 1B shows a spike trace from an example SHR cell; note the initial spike burst, with some baseline elevation because of fast simultaneous secretory events. In addition, note the long-lasting duration of spike activity for near 20 s, despite the fact that the ACh pulse duration lasted for only 2 s. This drastic difference of burst duration was better seen when the cumulative secretion versus time was plotted (Fig. 1C). The response of the control cell saturated after approximately 6 s, whereas that of SHR cells began to saturate after 18 s.

The integrated secretion (area in pC of all spikes secreted by each ACh pulse) is plotted in Fig. 1D; this secretion was approximately 2.6-fold higher in SHR compared with control cells. Because the spike number in an ACh pulse response was also in this range (approximately 2.8-fold higher; Fig. 1E), it seemed logical to conclude that the mean quantal size of single spike events should be similar; however, it was near 3-fold higher in SHRs compared with control cells (Fig. 1F). This may be explained by spike overlapping because of a faster secretion in SHR cells; in fact, complex spikes having three to four "fused" spikes are 3.5-fold more frequent in SHRs compared with control cells (Miranda-Ferreira et al., 2008).

A picture similar to ACh was found in cells stimulated with high K^+ pulses (70 K^+, 2 s). In the example control cell shown in Fig. 2A, the K^+ pulse elicited a spike burst with a delay of only 0.2 s. The secretory response lasted for 4 to 5 s. Again, the SHR cell responded with a secretory activity (Fig. 2C) that did not reach saturation even after 20 s of the K^+ pulse (see the cumulative secretion plot in Fig. 2B). The total secretion (Fig. 2D) and spike number (Fig. 2E) were approximately 2.5-fold higher in SHR cells. Once more, the quantal size of single vesicle events was 3-fold higher (Fig. 2F), indicating a faster secretion and vesicle overlapping in SHRs, with respect to control cells.

The Quantal Catecholamine Release Responses Triggered by CRT or FCCP in Chromaffin Cells from Normotensive and Hypertensive Rats. We previously have used CRT, a mixture of caffeine (20 mM), ryanodine (10 μM), and thapsigargin (1 μM), to cause a rapid release of ER Ca^{2+} into the cytosol and a subsequent irreversible ER Ca^{2+} depletion in bovine chromaffin cells (Cuchillo-Ibáñez et al., 2002). Here, we have used CRT acutely to see whether the sudden release of Ca^{2+} into the cytosol evoked by such drastic treatment could elicit similar or different secretory responses in control and SHR cells. We first applied two ACh or K^+ pulses, and these pulses elicited secretory responses similar to those of Figs. 1 and 2; hence, they are not shown here. In the example control cell of Fig. 3A, CRT caused spike activity after a delay of 2 s. Such activity ceased after approximately 7 s despite the continued presence of CRT. The secretion pattern in the SHR cell evoked by CRT lasted for approximately 30 s (Fig. 3B). Total secretion in control cells was around 140 pC and 2.2-fold higher in SHR cells (Fig. 3D). The spike number was approximately 2.4-fold higher in SHRs (Fig. 3E), and the single-vesicle quantal size was 3-fold higher (Fig. 3F). Thus, the differences in secretory patterns between control and SHR cells were similar with CRT (ER Ca^{2+} release) and ACh or K^+ (Ca^{2+} entry from the extracellular milieu). This is clearly seen upon comparison of Figs. 1C, 2C, and 3C.

The mitochondrial protonophores carbonylcyanide chloromethoxy phenylhydrazone and FCCP have been shown previously to augment the secretory responses evoked by ACh or K^+ pulses in populations of perifused bovine adrenal medulla chromaffin cells (Montero et al., 2000; Cuchillo-Ibáñez et al., 2002, 2003, 2004; Villalobos et al., 2002b) and in single voltage-clamped bovine chromaffin cells stimulated with depolarizing pulses or action potential waveforms (Giovannucci et al., 1999). Thus, in the present experiments, we used FCCP to study its effects on secretion.

Figure 4A shows that in a control cell, FCCP (1 μM) produced a large burst of secretory spikes that lasted for approximately 8 s; the cell became silent thereafter, despite the fact that FCCP was present for 30 s. After a 1-s delay, FCCP also caused a fast secretory activity in the example SHR cell shown in Fig. 4B.

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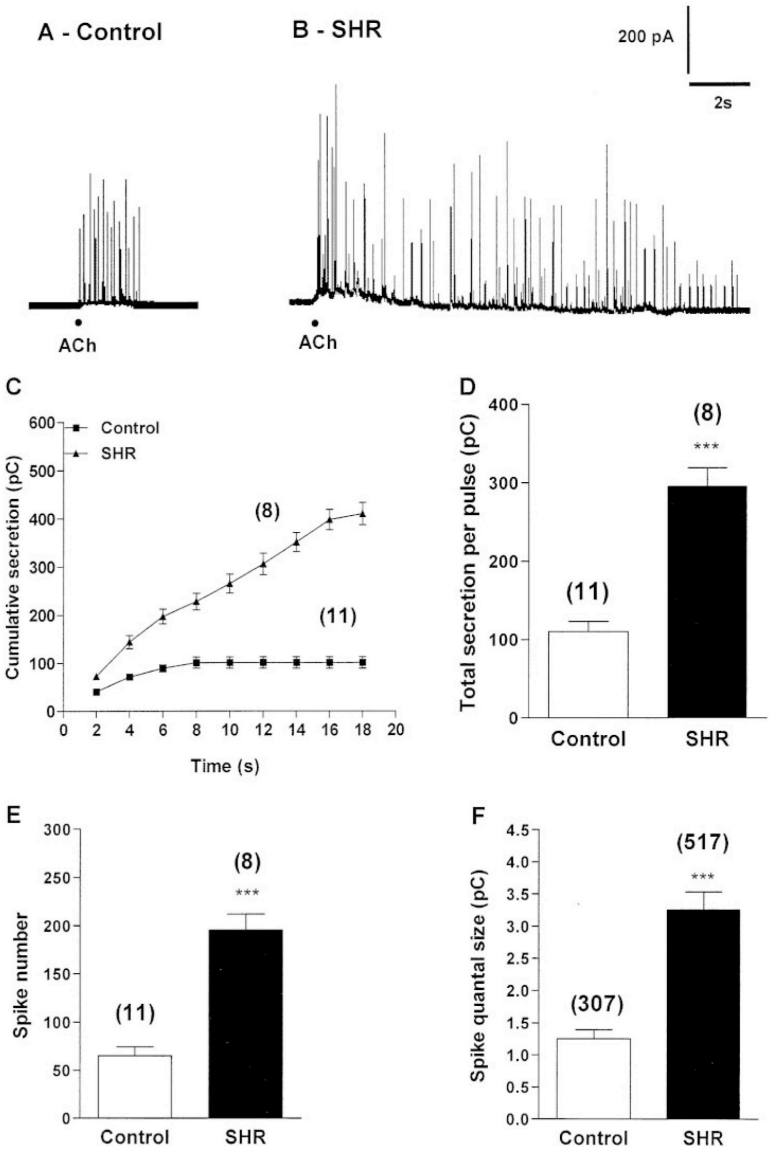

Fig. 1. ACh pulses produced longer lasting quantal catecholamine secretory responses in SHR chromaffin cells, compared with control cells. A, example record obtained from a control cell stimulated with an ACh pulse (1 mM, 2 s) as indicated by the dot at the bottom. B, record obtained from an example SHR cell, similarly stimulated with an ACh pulse. C, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the area of spikes is expressed and represented in pC (ordinate) as a function of time (abscissa). D, secretion per pulse (integrated area of all spikes generated by the ACh pulse) in pC, ordinate. E, total number of spikes secreted per each ACh pulse (ordinate). F, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means ± S.E. of the number of cells shown in parentheses on top of each column, from at least three different cultures. Data in F are means ± S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of D and E. ***, $p < 0.001$, with respect to control cells.

The duration of such response lasted for approximately 30 s, as shown in Fig. 4B. The total quantal secretion elicited by FCCP in control cells was 200 pC, more than double the secretion achieved by ACh and K^+ and 50% higher than the response to CRT. Total secretion in SHR cells reached 350 pC, approximately 20% above the responses elicited by the other three secretagogues. Spike number followed a similar pattern, 100 spikes in control cells and 255 in SHR cells (Fig. 4E). The quantal size of the individual spikes triggered by FCCP in control cells was 1.7 pC and in SHR cells reached as much as 3.5 pC. In conclusion, with some minor differences, the pattern of the secretion responses triggered by FCCP was similar to that of the other three secretagogues.

The Quantal Catecholamine Release Responses to ACh and K^+ Pulses in Control and SHR Cells That Had Been Pretreated with CRT or FCCP. As described above, CRT and FCCP caused by themselves a burst of quantal secretory spikes. Such secretory activity lasted 4 to 5 s in control cells and 20 to 30 s in SHR cells. This transient response could be due to cell damage. Thus, we decided to treat the cells for a 90-s period with CRT or FCCP, and at the end of this period, when basal secretory activity was absent, an ACh or K^+ pulse was applied. With this protocol, we sought two pieces of information: 1) to know whether after these strong drug pretreatments, the secretory machine was still viable; and 2) to determine how the suppression of the

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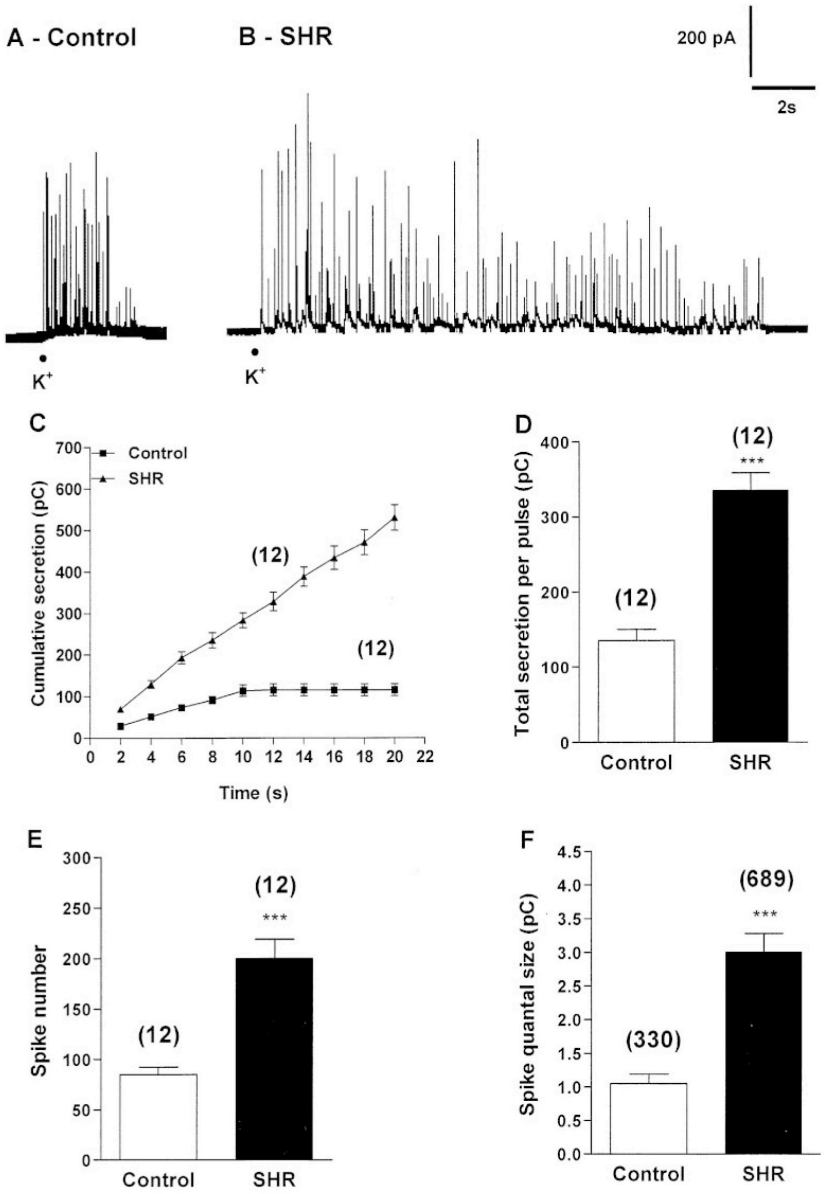

Fig. 2. K^+ pulses produced a longer lasting burst of quantal secretory spikes in SHR chromaffin cells, compared with control cells. A, example record obtained from a control cell stimulated with a K^+ pulse (70 mM K^+, 2 s) as indicated by the dot at the bottom. B, record obtained from an example SHR cell, similarly stimulated with a K^+ pulse. C, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the integrated area of spikes found in 2-s periods was cumulatively added and represented in pC (ordinate) as a function of time (abscissa). D, total secretion per pulse (integrated area of all spikes generated by the K^+ pulse) in pC, ordinate. E, total number of spikes secreted per each K^+ pulse (ordinate). F, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means ± S.E. of the number of cells shown in parentheses on top of each column, from at least three different cultures. Data in F are means ± S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of D and E. ***, $p < 0.001$, with respect to control cells.

ability of ER and mitochondria to take up Ca^{2+} from the cytosol and to release Ca^{2+} back into the cytosol could affect the responses to ACh and K^+. It is firmly established that CRT suppresses the capacity of ER to handle Ca^{2+}, and FCCP suppresses the ability of mitochondria to handle such cation (Alonso et al., 1999; Montero et al., 2000; Cuchillo-Ibáñez et al., 2002, 2004; Villalobos et al., 2002b).

With this protocol, we observed that CRT pretreatment did not deteriorate the secretory responses to ACh or K^+; on the contrary, such responses were more than doubled after CRT treatment of both control and SHR cells (Fig. 5). FCCP was even more efficacious than CRT in causing an augmentation of ACh- and K^+-elicited responses. For instance, in control cells, FCCP augmented 4.5-fold the ACh response in control cells (Fig. 6A) and near 3-fold in SHR cells (Fig. 6B). The K^+ responses were augmented 3-fold in control cells (Fig. 6C) and 2.3-fold in SHR cells (Fig. 6D).

In a few control and SHR cells, we performed experiments with pretreatment with the combined CRT and FCCP. We did not observe a further enhancement of secretion. In fact, the cells responded to ACh and K^+ with poorer responses, indicating that this drastic treatment could damage the cells.

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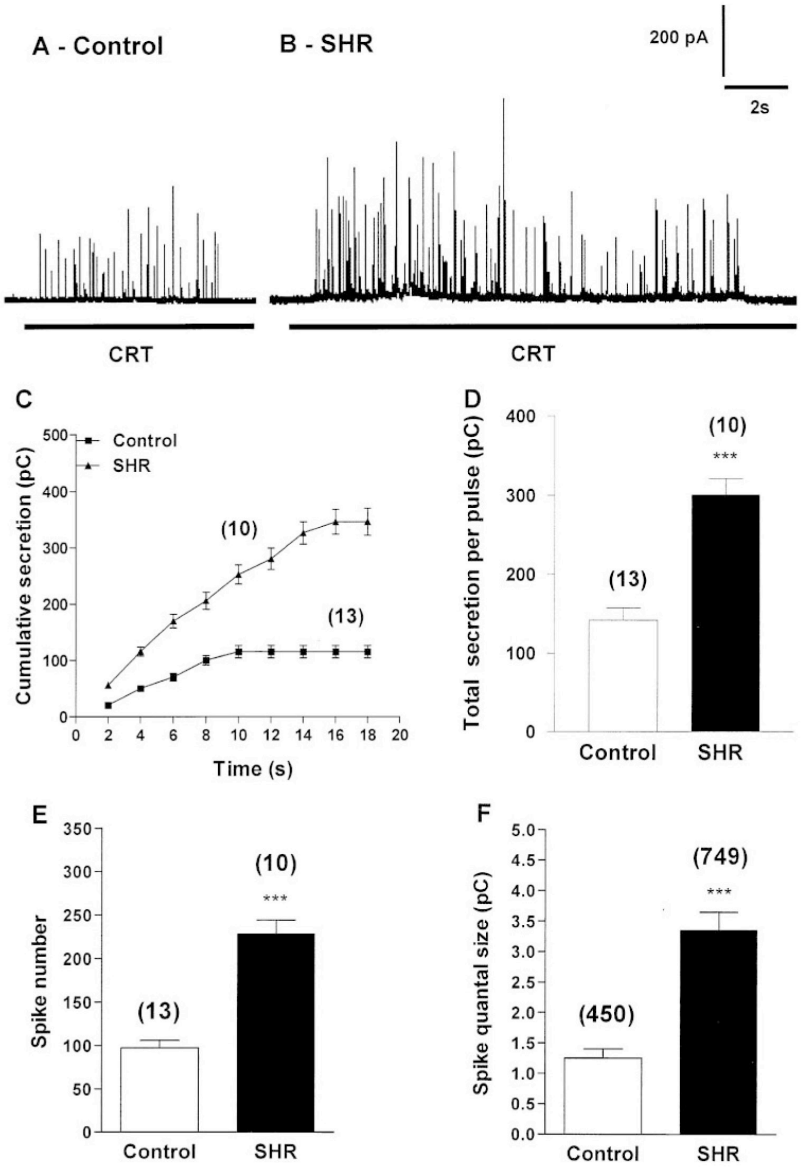

Fig. 3. CRT produced a longer lasting burst of quantal secretory spikes in SHR chromaffin cells, compared with control cells. A, example record obtained from a control cell stimulated with CRT (a mixture of 20 mM caffeine, 10 μM ryanodine, and 1 μM thapsigargin) as indicated by the horizontal bar at the bottom. B, record obtained from an example SHR cell, similarly stimulated with CRT. C, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the integrated area of spikes found in 2-s periods was cumulatively added and represented in pC (ordinate) as a function of time (abscissa). D, total secretion (integrated area of all spikes generated during CRT treatment) in pC, ordinate. E, total number of spikes secreted during CRT treatment (ordinate). F, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means ± S.E. of the number of cells shown in parentheses on top of each column, from at least three different cultures. Data in F are means ± S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of D and E. ***, $p < 0.001$, with respect to control cells.

Kinetics of Individual Quantal Spike Events Released by ACh, K^+, CRT, or FCCP. As shown in Figs. 1 to 4, the quantal size of individual secretory events released from control cells is approximately 1 pC, and that for SHR cells is 3-fold higher (Figs. 1–4). We wanted to know more approximately the kinetic features of control and SHR single spikes and analyzed other parameters such as: 1) $t_{1/2}$, an indication of spike width; 2) t_{max}, the time to peak, an indication of the spike activation rate; and 3) I_{max}, the spike amplitude.

Table 1 shows that the ACh-evoked secretory events in control cells had a $t_{1/2}$ of 8.9 ms, a t_{max} of 12.5 ms, and an I_{max} of 155 pA. In SHR cells, these parameters were as follows: $t_{1/2}$ 7.5 ms, 20% lower; t_{max} 8.5 ms, 30% lower; and I_{max}, 283 pA, 45% higher. These changes indicate that the spikes of SHR cells were narrower, faster, and of higher amplitude, with respect to those of control cells. Their 3-fold greater quantal content of catecholamines, expressed by spike area, gave rise to larger and sharper spikes.

The K^+-elicited spikes were in the range of those evoked by ACh, the SHR cells having a $t_{1/2}$ and t_{max} 20 to 25% lower and an I_{max} 50% higher than control cells. Similar results were obtained for the kinetics of spikes generated by cell stimulation with CRT or FCCP. Thus, it seems that these kinetic differences are due to variations in the intrinsic properties of control and SHR cells (i.e., greater quantal size and more vesicles available for release) rather than to the type of

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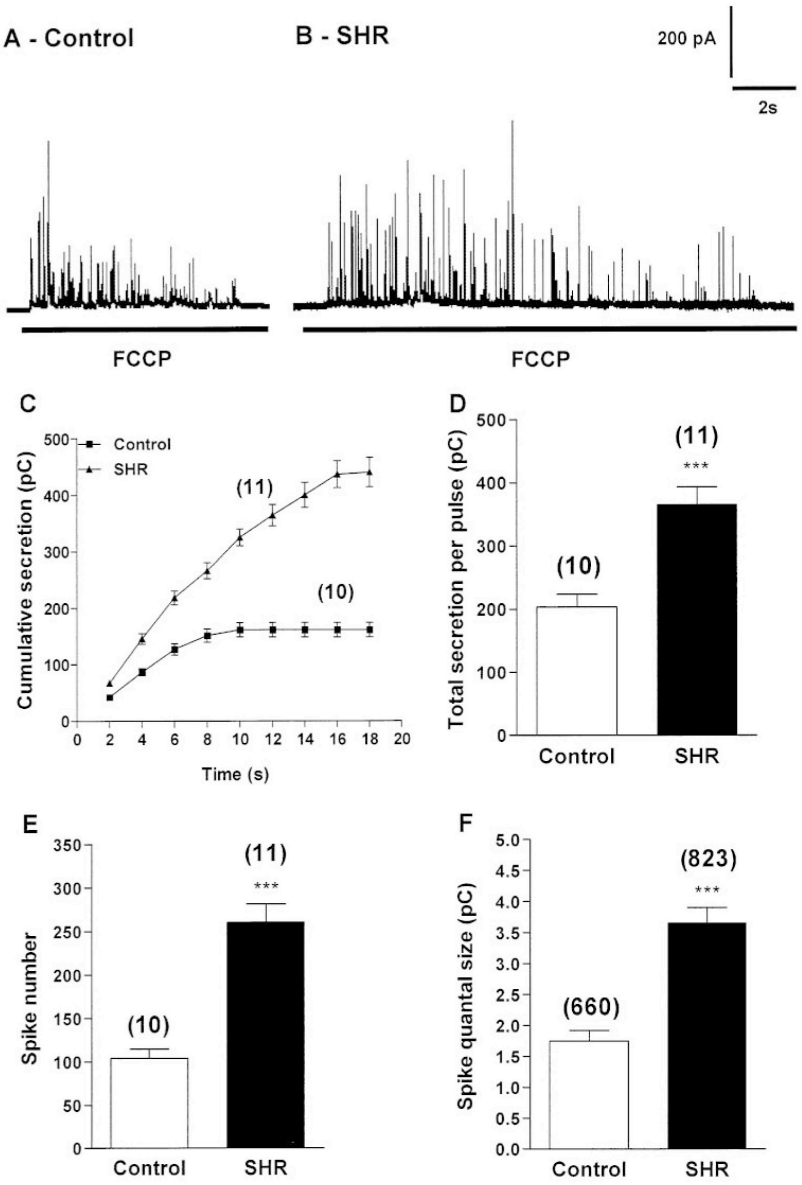

Fig. 4. FCCP produced a longer lasting burst of quantal secretory spikes in SHR chromaffin cells, compared with control cells. A, example record obtained from a control cell stimulated with FCCP (1 μM) as indicated by the horizontal bar at the bottom. B, record obtained from an example SHR cell, similarly stimulated with FCCP. C, cumulative secretion, calculated at 2-s intervals in traces similar to those shown in A and B; the integrated area of spikes found in 2-s periods was cumulatively added and represented in pC (ordinate) as a function of time (abscissa). D, total secretion (integrated area of all spikes generated during FCCP treatment) in pC, ordinate. E, total number of spikes secreted during FCCP treatment (ordinate). F, quantal content of individual secretory events, expressed in pC (ordinate). Data in D and E are means ± S.E. of the number of cells shown in parentheses on top of each column, from at least three different cultures. Data in F are means ± S.E. of the number of individual spikes shown in parentheses on top of each column; those spikes are from the experiments of D and E. ***, $p < 0.001$, with respect to control cells.

secretagogue used to stimulate secretion by Ca^{2+} entry (ACh or K^+) or by Ca^{2+} release and blockade of Ca^{2+} fluxes at intracellular organelles (CRT or FCCP).

Discussion

In this study, we have found drastic differences in the secretion kinetics of adrenal medulla chromaffin cells from control normotensive rats and those isolated from hypertensive animals. Such differences of SHRs with respect to control cells can be summarized as follows: 1) a longer lasting burst of secretory spikes, 2) a greater cumulative burst secretion per stimulus, 3) a 3-fold higher quantal content of individual secretory spikes, and 4) a faster secretory response of individual vesicles. Overall, this pattern suggests that in SHRs, the adrenal gland could respond with a sharper and prolonged release of catecholamines into the circulation upon stressful conflicts.

In a recent study, we compared the patterns of secretory responses elicited by ACh and K^+ and concluded that: "the much greater catecholamine release responses in SHRs are explained by faster exocytosis of more vesicles, with greater

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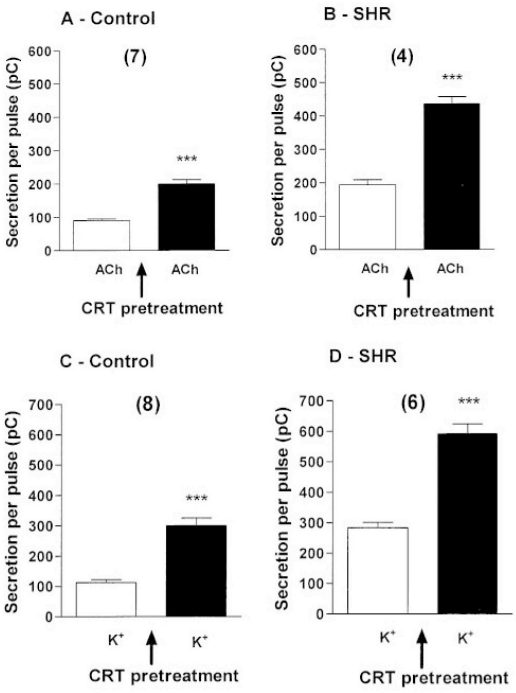

Fig. 5. Quantal catecholamine release responses evoked by ACh or K^+ pulses in control and SHR cells pretreated with CRT. Cells were initially stimulated with an ACh (1 mM, 2 s) or K^+ (70 mM, 2 s) pulse. Cells were then perifused with basal Krebs-HEPES solution for 3.5 min and subsequently with CRT, a mixture of 20 mM caffeine, 10 μM ryanodine, and 1 μM thapsigargin, to cause ER Ca^{2+} depletion and to irreversibly block ER Ca^{2+} fluxes, for 1.5 min. Once silent, the cell was challenged with a second ACh or K^+ pulse. Total secretion per pulse (integrated area of all spikes generated by each one of the two ACh or K^+ pulses) is shown in the ordinates. A and B, control and SHR cells stimulated with ACh, before and after CRT treatment; C and D, control and SHR cells stimulated with K^+. Data are means ± S.E. of the number of cells shown in parentheses on top each column from at least three different cultures. ***, $p < 0.001$, with respect to the response obtained before CRT treatment.

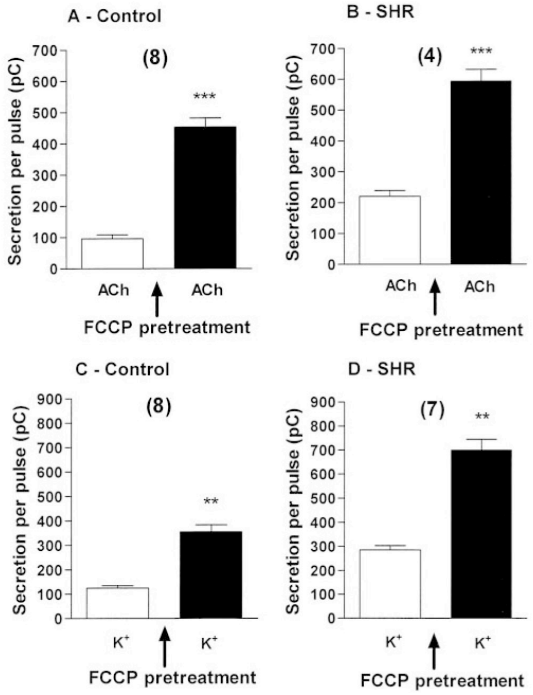

Fig. 6. Quantal catecholamine release responses evoked by ACh or K^+ pulses in control and SHR cells pretreated with FCCP. Cells were initially stimulated with an ACh (1 mM, 2 s) or K^+ (70 mM, 2 s) pulse. Cells were then perifused with basal Krebs-HEPES solution for 3.5 min and subsequently with FCCP (1 μM) to block mitochondrial Ca^{2+} fluxes for 1.5 min. Once silent, the cell was challenged with a second ACh or K^+ pulse. Total secretion per pulse (integrated area of all spikes generated by each one of the two ACh or K^+ pulses) is shown in the ordinates. A and B, control and SHR cells stimulated with ACh, before and after FCCP treatment; C and D, control and SHR cells stimulated with K^+. Data are means ± S.E. of the number of cells shown in parentheses on top of each column from at least three different cultures. **, $p < 0.01$; ***, $p < 0.001$ with respect to the response obtained before FCCP treatment.

quantal catecholamine content, during a much longer secretory period." The present study corroborates this conclusion and adds novel elements on the implication of an alteration of catecholamine secretory mechanisms to the understanding of the pathogenesis of hypertension. Thus, an alteration of the Ca^{2+} handling by the ER and mitochondria could also alter the secretory responses and explain their differences between control and SHR cells. In fact, alterations of intracellular Ca^{2+} homeostatic mechanisms occur in vascular smooth muscle cells in SHRs. For instance, a large Ca^{2+} influx and a high basal $[Ca^{2+}]_c$ have been seen during rest in conduit arteries (i.e., the aorta and carotid and femoral arteries) from SHRs compared with normotensive rats (Jelicks and Gupta, 1990; Asano et al., 1996). This has also been found in small mesenteric arteries from SHRs, where the sarcoplasmic reticulum has a larger capacity for Ca^{2+} storage (Nomura and Asano, 2002).

Donohue et al. (1988) found an enhanced norepinephrine release from different tissues with rich sympathetic innervation in SHRs, with respect to normotensive rats. More recently, enhanced catecholamine release also has been shown in perfused adrenal glands (Lim et al., 2002a) or single adrenal chromaffin cells of SHRs, compared with control animals (Miranda-Ferreira et al., 2008). Such enhanced release could be due to different Ca^{2+} entry pathways or to differences in the Ca^{2+} redistribution into, and Ca^{2+} release from, the ER and mitochondria. In fact, a functional triad controlling Ca^{2+} and exocytotic signals is present in adrenal chromaffin cells, as revealed through the use of ER-targeted

TABLE 1
Kinetic parameters of individual secretory events released from control and SHR cells, from the experiments shown in Figs. 1 to 4

Stimulus	Type of Chromaffin Cell	$t_{1/2}$	t_{max}	I_{max}	n
		ms		*pA*	
ACh	Control	8.9 ± 0.3	12.5 ± 0.5	155 ± 7.6	307
	SHR	7.5 ± 0.4*	8.5 ± 0.8***	283 ± 9.1***	517
K^+	Control	7.2 ± 0.3	11.3 ± 0.7	130 ± 6.3	330
	SHR	6.3 ± 0.2*	9.2 ± 0.5*	260 ± 7.3***	689
CRT	Control	7.8 ± 0.4	8.5 ± 0.3	105 ± 6	450
	SHR	6.0 ± 0.3***	6.8 ± 0.4***	161 ± 9***	749
FCCP	Control	7.1 ± 0.4	8.2 ± 0.2	137 ± 8	660
	SHR	5.4 ± 0.3***	6.5 ± 0.4***	171 ± 10***	823

aequorin (Alonso et al., 1999) or mitochondria-targeted aequorin (Montero et al., 2000; Villalobos et al., 2002a). Such a triad is formed by high-voltage activated Ca^{2+} channels, the ER, and mitochondria located nearby the plasmalemma. The triad will control Ca^{2+} entry and its subsequent intracellular redistribution, shaping in this manner the local $[Ca^{2+}]_c$ transients, vesicle traffic, and exocytotic responses generated by cell activation with depolarizing pulses of ACh or high K^+ (Garcia et al., 2006). Mitochondria also play a relevant role in the clearance of the $[Ca^{2+}]_c$ elevations elicited by stimulation of rat chromaffin cells (Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997). On the other hand, ER Ca^{2+} release by histamine or muscarine causes the release of catecholamines (Wakade, 1981), suggesting that as in bovine chromaffin cells, rat chromaffin cells could also have a functional triad to control $[Ca^{2+}]_c$ and exocytotic signals.

In our present investigation, we have seen that the secretory responses to ACh and K^+ were similar, indicating that differences in Ca^{2+} entry during nicotinic receptor activation or direct cell depolarization did not account for the greater responses of SHR cells. Furthermore, CRT and FCCP caused secretory responses that were qualitatively and quantitatively similar, also suggesting that differences in intracellular ER Ca^{2+} release from the ER or mitochondria did not explain the much greater responses in SHRs compared with control cells. In addition, control and SHR cells with their ER depleted by CRT pretreatment or with blockade of mitochondrial Ca^{2+} uptake through their uniporter elicited by FCCP pretreatment responded with similar enhanced responses to ACh and K^+. This indicates that differences in Ca^{2+} redistribution into the ER and mitochondria and/or its release from these organelles do not explain the much greater secretory responses seen in SHRs with respect to control cells, in the different experimental conditions studied. However, ER Ca^{2+} release is known to be taken up by mitochondria in chromaffin cells (Montero et al., 2000); thus, the possibility exists that this "Ca^{2+} shuttle" is altered in SHRs compared with control cells. So, pending a more direct study on $[Ca^{2+}]_c$ transients and their clearance, as that performed in the Bertill Hille laboratory in normotensive rats (Herrington et al., 1996; Park et al., 1996; Babcock et al., 1997), our quantal secretion experiments indicate that the functional triad that control Ca^{2+} homeostasis in chromaffin cells (Garcia et al., 2006) is not altered in SHR chromaffin cells.

Rather, differences in the size of the docked vesicle pool underneath the plasmalemma (Neher, 1998) and in the kinetics of single-vesicle exocytosis could explain the drastic differences in the secretory responses obtained in control and SHR cells. First, the quantal size of individual vesicles was as much as 3-fold higher in SHRs compared with control cells; this was true for all protocols used to stimulate secretion i.e., ACh, K^+, CRT, FCCP, or ACh and K^+ after cell pretreatment with CRT or FCCP. The obvious conclusion from these data is that at least 3-fold greater catecholamine secretion will be seen in SHR cells. Second, the quantal content of integrated burst spikes was also 2- to 4-fold greater in SHR cells. Third, the individual amperometric events had a faster kinetics. Overall, these properties suggest that upon a given stimulus, the adrenal medulla in the intact animal will respond much more quickly and with a more prolonged response in SHRs than in normotensive rats. This seems to derive from differences in the structure and function of the secretory machinery, rather than from differences in Ca^{2+} handling by intracellular organelles. It will be interesting to perform an electron microscopic study to find out whether the number of chromaffin vesicles and their disposition at subplasmalemmal sites are different in control and SHR chromaffin cells. These types of morphological studies may provide information on the size of different vesicle pools nearby the secretory machinery (Vitale et al., 1995). Another interesting question is related to the possibility that the enhanced norepinephrine release from sympathetic nerve terminals in SHRs compared with normotensive rats (Donohue et al., 1988) could also underlie a mechanism similar to that described here for chromaffin cells.

An additional relevant issue in this study concerns the controversy on the role of the ER Ca^{2+} store in controlling the release of catecholamines. Concerning the effect of ER Ca^{2+} depletion on depolarization-evoked secretion, three studies are available in bovine chromaffin cells. Mollard et al. (1995) showed no effect, Pan and Fox (2000) showed potentiation followed by depression, and Cuchillo-Ibáñez et al. (2002) showed a pronounced depression of ACh responses but no effect on K^+-evoked secretory responses. Here, we have shown that under ER Ca^{2+} depletion, the ACh and K^+ responses were drastically augmented in both control and SHR cells. This may be explained by an augmentation of vesicle traffic because of Ca^{2+} release from the ER during the CRT treatment period preceding the ACh or K^+ pulses; this will lead to accumulation of more vesicles underneath the plasma membrane, as suggested by von Rüden and Neher (1993) to be the case upon stimulation of ER Ca^{2+} release with histamine.

In conclusion, we have found that the quantal secretion of catecholamines from chromaffin cells is higher and of longer duration in SHRs compared with control rats. This was true for the release triggered by Ca^{2+} entry (ACh or K^+ stimulation) or by Ca^{2+} release from the ER (CRT) or the mitochondrion (FCCP). Furthermore, the individual amperometric events are faster and have a 3-fold higher quantal size in SHRs compared with control rats. We believe that these data are valuable for the understanding of the pathogenesis of hypertension. In addition, they might provide clues to identify new targets for the development of novel antihypertensive compounds acting on the exocytotic machinery.

Acknowledgments

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Address correspondence to: Dr. Antonio G. García, Instituto Teófilo Hernando de I+D del Medicamento, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo 4, 28029 Madrid, Spain. E-mail: agg@uam.es

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Greater cytosolic and mitochondrial calcium transients in adrenal medullary slices of hypertensive, compared with normotensive rats

Regiane Miranda-Ferreira [a], Ricardo de Pascual [b,c], Soraya S. Smaili [a], Afonso Caricati-Neto [a], Luis Gandía [b,c], Antonio G. García [b,c,d,*], Aron Jurkiewicz [a]

[a] Departamento de Farmacología, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil
[b] Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, C/ Arzobispo Morcillo, 4, 28029 Madrid, Spain
[c] Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, C/ Arzobispo Morcillo, 4, 28029 Madrid, Spain
[d] Servicio de Farmacología Clínica, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, C/ Diego de León, 62, 28006 Madrid, Spain

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ABSTRACT

Pronounced differences in the kinetics of single-vesicle catecholamine release from adrenal chromaffin cells stimulated with acetylcholine or high potassium (K^+) have been recently found between normotensive Wistar rats (NWRs) and spontaneously hypertensive rats (SHRs). Such differences could be explained on the basis of distinct mechanisms of calcium (Ca^{2+}) handling by chromaffin cells of NWRs and SHRs. We have explored here this hypothesis in adrenal medullary slices loaded with calcium fluorescent probes to measure the changes in Ca^{2+} concentration in the cytosol ($[Ca^{2+}]_c$), endoplasmic reticulum ($[Ca^{2+}]_{er}$), and mitochondria ($[Ca^{2+}]_m$). We found the following differences on calcium handling in SHRs, as compared with NWR: (i) higher basal $[Ca^{2+}]_c$ and basal $[Ca^{2+}]_m$; (ii) greater $[Ca^{2+}]_c$ elevations elicited by acetylcholine and K^+, with faster activation but slower inactivation; (iii) greater $[Ca^{2+}]_c$ elevations elicited by CRT (a mixture of caffeine, ryanodine, and thapsigargin) and by the mitochondrial protonophore FCCP (carbonylcyanide p-(trifluoromethoxy) phenylhydrazone). The higher basal $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ suggest an enhanced mitochondrial Ca^{2+} uptake, and the greater $[Ca^{2+}]_c$ elevations produced by FCCP indicates a higher mitochondrial Ca^{2+} release into the cytosol. This alteration of intracellular Ca^{2+} movements could explain the greater quantal catecholamine release responses seen in SHRs, as compared with NWRs in previous studies. Furthermore, enhanced mitochondrial Ca^{2+} cycling may be the basis for the dysfunction of mitochondrial bioenergetics, reported to be present in hypertensive states.

1. Introduction

Alterations in the activity of the sympathoadrenal axis and the subsequent rate of catecholamine release, have been implicated in the pathogenesis of essential hypertension, as proven by the following observations:1) classic drugs such as blockers of α- and β-adrenoceptors, reserpine, α-methyldopa, ganglionic blocking agents, or guanethidine, known to interfere with this axis, have been used to treat hypertension (Hoffman, 2006); 2) hypertensive patients have elevated sympathetic nerve activity, as revealed with microneurography (Anderson et al., 1989); and 3) plasma levels of norepinephrine and epinephrine are augmented in hypertensive patients (de Champlain et al., 1976; Goldstein et al., 1983) as well as in spontaneously hypertensive rats (SHR) (Grobecker et al., 1975; Iriuchijima, 1973; Pak, 1981). These enhanced circulating catecholamine levels are likely due to augmented catecholamine release from sympathetic neurons and adrenal chromaffin cells (Arnaiz et al., 1978). In fact, Lim et al. (2002) have shown a greater catecholamine release in adrenal glands from SHRs, compared with normotensive rats.

We recently performed two studies on the kinetics of single-vesicle secretory events, using a carbon fiber electrode and amperometry in isolated single adrenal chromaffin cells from control and SHRs. We found that SHR chromaffin cells stimulated with short pulses of acetylcholine or K^+, had a more sustained production of spike secretory events and a drastic augmentation of the quantal catecholamine content of individual secretory vesicles with faster fusion kinetics, compared with normotensive rats (Miranda-Ferreira et al., 2008). In another study we used compounds that deplete the endoplasmic reticulum Ca^{2+} store or that block mitochondrial Ca^{2+} uptake. To this aims we used a mixture of caffeine, ryanodine and thapsigargin (CRT) to cause a rapid depletion of the endoplasmic reticulum Ca^{2+} store (Cuchillo-Ibanez et al., 2002), and the protonophore FCCP (carbonylcyanide p-trifluoromethoxy phenylhydrazone) to release any Ca^{2+} stored in mitochondria, and to prevent mitochondrial Ca^{2+} uptake during cell stimulation (Montero et al.,

* Corresponding author. Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 4, 28029, Madrid, Spain. Tel./fax: +34 91 497 31 20.
E-mail address: agg@uam.es (A.G. García).

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2000). We observed that 1) secretory responses lasted longer in SHRs compared with controls; 2) the responses to acetylcholine and K^+ were markedly enhanced in cells previously treated with CRT or FCCP; and 3) the individual single-vesicle secretory spikes were faster and had a greater quantal size in SHRs compared with control rats (Miranda-Ferreira et al., 2009).

In the light of these differences on quantal catecholamine release, we felt of interest to explore the question of whether they could be explained by differences in the elevations of cytosolic $[Ca^{2+}]_c$ elicited by stimulation of chromaffin cells with acetylcholine, K^+, CRT, or FCCP, in adrenal slices of normotensive (NWRs) and hypertensive rats (SHRs). We used here freshly prepared adrenal slices because they are more relevant from physiological and pathophysiological points of view. We have found that in SHR slice chromaffin cells, basal $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ were substantially higher than those found in preparations from normotensive rats. We also found higher $[Ca^{2+}]_c$ elevations in SHR chromaffin cells stimulated with acetylcholine, K^+, CRT or FCCP.

2. Materials and methods

2.1. Animals

Animals were manipulated according to the guidance of the Ethics Committee for Handling Research Animals of the Federal University of São Paulo (São Paulo, Brazil). Male 16-week-old normotensive Wistar rats (NWR) and Spontaneously Hypertensive Rats (SHR) weighing approximately 300 g were housed at 24 ± 2 °C, with 60% relative humidity, on a 12 h light/dark cycle. Animals were fed a standard diet and water ad libitum and periodically weighted.

2.2. Preparation of adrenal medullary slices

Animals were sacrificed by mean of decapitation and their adrenal glands were removed and immediately immersed in a Tyrode nutrient solution with the following composition (in mM): 137 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 1 $MgCl_2$; 12 $NaHCO_3$; 0.36 KH_2PO_4; 11 glucose. The pH was adjusted at 7.3 with 95% O_2/5% CO_2. Adrenal glands were cleaned of fat, embedded in agarose (4%), and mounted in a vibratome chamber (1000 Plus Sectioning System, Campden Instruments, INC., U.S.A.) and cut in slices of 200 μm thickness. After incubation with Ca^{2+} indicators, the slices were mounted in a Leiden chamber of a fluorescence microscope (Nikon TE 300, Nikon, Osaka, Japan) coupled to a CCD camera (Quantix 512 — Roper Scientific Inc, Princeton Instruments, NJ, U.S.A.) using the Spectralyser software (Paul Anderson, NJ, U.S.A.) to select a field containing chromaffin cells in the intact adrenal medullary tissue.

2.3. Cytosolic Ca^{2+} measurements

To study $[Ca^{2+}]_c$ changes, slices were incubated with 10 μM of the ratiometric dye fura-2.AM (Grynkiewicz et al., 1985) in a Tyrode solution containing 0.01% pluronic acid F-127 at room temperature for 45 min; this solution was oxygenated and protected from light. After incubation, slices were washed and mounted in a Leiden chamber with 1 ml of Tyrode solution and kept at 37 °C during the experiments. Basal $[Ca^{2+}]_c$ was measured during the first 10 images, which were taken at 5-s intervals. Calibration was done by adding 1 mM digitonin (maximal fura-2 fluorescence, R_{max}).

Chromaffin cells in the adrenal medulla slices were challenged with acetylcholine (100 μM) or KCl (70 mM), to stimulate Ca^{2+} influx through VDCCs. CRT, a mixture of caffeine (20 or 50 mM), ryanodine (10 μM) and thapsigargin (2 μM) was used to study endoplasmic reticulum Ca^{2+} release. The protonophore FCCP (carbonylcyanide p-(trifluoromethoxy) phenylhydrazone, 5 μM) was used to study Ca^{2+} release from mitochondria. We also studied the simultaneous Ca^{2+} release from endoplasmic reticulum and mitochondria by adding a solution containing CRT and FCCP in combination.

2.4. Endoplasmic reticulum Ca^{2+} measurements

To study Ca^{2+} from the endoplasmic reticulum compartment, slices were incubated with Fura-FF.AM (10 μM), a ratiometric dye that presents more affinity for the endoplasmic reticulum if incubated longer at 37 °C (Hajnoczky et al., 1999; Hajnoczky and Thomas, 1997; London et al., 1994). Slices were incubated in a Tyrode solution containing Fura-FF.AM and 0.01% pluronic acid F-127 at 37 °C for 90 min, oxygenated with 95% O_2/5% CO_2 and protected from light. After incubation with Fura-FF, slices were bathed with Ca^{2+}-free medium and digitonin (5 μM, 30 s), in order to cause a semi-permeabilisation of the plasma membrane to facilitate release of indicator that was not confined to the endoplasmic reticulum compartment. The Ca^{2+}-free medium solution ("intracellular medium") contained (in mM): 10 NaCl, 120 KCl, 1 KH_2PO_4, 20 HEPES, and 2 EGTA; this solution was supplemented with 1 mM ATP, 10 μM creatine phosphate, 10 μM creatine phosphate kinase and 2 mM pyruvate, in order to restore the energy expenditure caused by cell semi-permeabilisation. Calibration was done by addition of ionomycin (1 mM), a membrane permeant ionophore that allows find out the minimal fluorescence (R_{min}).

To check endoplasmic reticulum Ca^{2+} variations we studied the effects of a CRT solution containing 20 mM or 50 mM caffeine (C_{20}RT, C_{50}RT), to stimulate Ca^{2+} release from the endoplasmic reticulum. We also studied the cross-talking of endoplasmic reticulum and mitochondrial Ca^{2+} by using FCCP to stimulate the leak of Ca^{2+} from mitochondria and its uptake by the endoplasmic reticulum.

2.5. Mitochondrial Ca^{2+} measurements

To study Ca^{2+} movements in the mitochondrial compartment, slices were incubated with Rhod-2.AM (10 μM), an indicator that presents a higher affinity for mitochondria (Babcock et al., 1997; Golovina and Blaustein, 1997; Hoth et al., 1997). Slices were incubated in a Tyrode solution containing Rhod-2.AM plus 0.01% pluronic acid F-127 at 37 °C for 60 min, oxygenated and protected from light. After incubation with Rhod-2, slices were bathed with Ca^{2+}-free medium and digitonin (5 μM, 30 s) to produce a semi-permeabilisation of the plasma membrane to assure the washout of the indicator not confined to the mitochondrial compartment. The Ca^{2+}-free medium (intracellular medium) contained (in mM): 35 NaCl, 115 KCl, 1 KH_2PO_4, 20 HEPES, 2 EGTA, pH 7.2; this solution was supplemented with 1 mM ATP, 10 μM creatine phosphate, 10 μM creatine phosphate kinase, and 2 mM pyruvate to restore the energy expenditure caused by the cell semi-permeabilisation. Calibration was done by addition of 1 mM ionomycin, a membrane permeant that allows finding out minimal fluorescence at the end of the experiment (Rmin).

To study the changes of $[Ca^{2+}]_m$ we studied the effects of FCCP to stimulate the release of Ca^{2+} from mitochondria. We also studied the cross-talking of mitochondria and the endoplasmic reticulum by using CRT to stimulate the leak of Ca^{2+} from endoplasmic reticulum and observe its uptake into mitochondria. Conversely, we also studied how the mitochondrial Ca^{2+} released by FCCP was taken up by the endoplasmic reticulum.

2.6. Materials and solutions

The following materials were used: acetylcholine chloride, FCCP, thapsigargin and ryanodine were from Sigma-Aldrich (St. Louis, MO). Fura-2-AM, Fura-FF.AM, Rhod-2.AM and pluronic acid were from Invitrogen-Molecular Probes (Carlsbad, CA). All other chemicals used were from were from Invitrogen (Brazil).

2.7. Statistical analysis

We normalized data by the $(F-F0)/F0\times100$, where $F0$ is the mean of basal $[Ca^{2+}]$ levels. In the graphs on $[Ca^{2+}]_c$ measurements, 0 was considered as baseline and 100% as R_{max}. In graphs for the measurement of $[Ca^{2+}]_{er}$ and $[Ca^{2+}]_m$, 100% was considered baseline (maximal Ca^{2+} of the compartments) and 0 was considered R_{min} (minimal Ca^{2+} from compartments) because of the Ca^{2+} leak after treatment. We analyzed the maximum and minimum Ca^{2+} changes during 10 min periods, considering the mean fluorescence as a function of time. When using Fura-2 and Fura-FF, the mean of fluorescence ratio was taken into account because these dyes are ratiometric (340 and 380 nm excitation wavelengths). For Rhod-2 we used direct fluorescence (540 nm excitation).

Data are means ± S.E.M. Differences were considered significant at $P<0.05$. Two-population (NWR and SHR) comparisons were made with Student t test. We also used ANOVA to compare responses to three different treatments (CRT, FCCP, CRT + FCCP) between NWR and SHR.

3. Results

3.1. Basal $[Ca^{2+}]$ in cytosol, endoplasmic reticulum, and mitochondria of chromaffin cells in adrenal slices of normotensive and hypertensive rats

Some studies have found an elevated basal $[Ca^{2+}]_c$ in resting conduit arteries, i.e. aorta, carotid, and femoral arteries from SHRs compared with NWRs (Asano et al., 1996; Jelicks and Gupta, 1990); this difference has also been found in small mesenteric arteries (Nomura and Asano, 2002). However, no data are available on the basal $[Ca^{2+}]_{er}$ nor $[Ca^{2+}]_m$ in these vessels, neither in chromaffin cells. Therefore, we studied here the basal $[Ca^{2+}]$ in such three compartments of chromaffin cells in adrenal slices of normotensive and hypertensive rats.

Slices were loaded with Fura-2, Fura-FF, or Rhod-2 to measure the variations of the $[Ca^{2+}]$ at the cytosol, endoplasmic reticulum, or mitochondria, respectively. Each individual experiment commenced with an initial recording period, to estimate the basal fluorescence. The slice was subsequently perifused with one of the stimulation solutions used in this study. With this protocol we gained information about the basal $[Ca^{2+}]$ in those three cell compartments, both in slices from NWRs and SHRs.

For instance, Fig. 1A shows original traces of fluorescence obtained in basal conditions before challenging the tissue with acetylcholine (100 μM), in Fura-2-loaded slices. The initial fluorescence trace, measured as the ratio 340/380 nm, was substantially higher in the SHR slice, as compared with the NWR slice. This was also true for the acetylcholine-elicited responses that will be described in more detail in the next section. Fig. 1B displays pooled results from 15 slices; the basal $[Ca^{2+}]_c$ was almost double in SHRs, as compared with NWRs.

The prototype experiment to measure the $[Ca^{2+}]_{er}$ variations is shown in Fig. 1C. The initial fluorescence ratio (R340/380) was 0.52 in the NWR slice and 0.58 in the SHR slice. In these traces, the effects of $C_{20}RT$ and $C_{50}RT$ causing ER Ca^{2+} depletion are also shown (see later). Fig. 1D shows pooled results of 10 slices; the basal $[Ca^{2+}]_{er}$ was similar in NWRs and SHRs.

Original fluorescence traces from an example slice loaded with Rhod-2 are shown in Fig. 1E. The basal fluorescence was 2.13-fold higher in SHR as compared with the NWR slice. Depletion of $[Ca^{2+}]_m$ elicited by FCCP is also shown in these example slices (see later). Fig. 1F shows pooled results on the basal $[Ca^{2+}]_m$, which was 2.25-fold higher in chromaffin cells from SHRs as compared with NWRs.

In summary, the basal $[Ca^{2+}]$ in the cytosol and mitochondria of chromaffin cells in SHR adrenal slices was substantially higher than the basal $[Ca^{2+}]$ found in NWR slices; in contrast, the basal $[Ca^{2+}]$ was similar in the endoplasmic reticulum of chromaffin cells from normotensive and hypertensive slices.

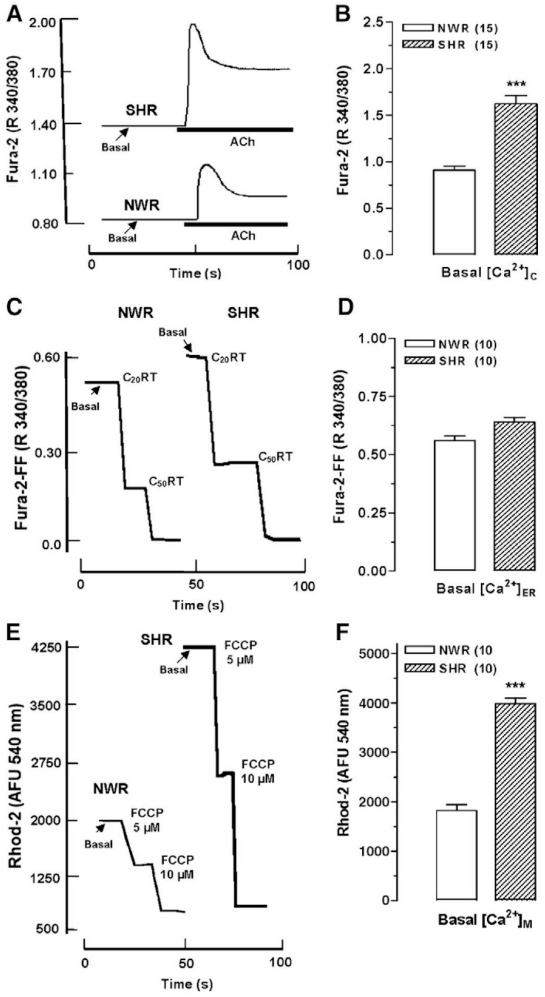

Fig. 1. Typical traces showing the experimental protocols followed in this study to measure basal and evoked $[Ca^{2+}]$ changes in different cell compartments. A, example traces obtained from Fura-2-loaded slices of chromaffin cells from adrenal medullae, to measure the $[Ca^{2+}]_c$ as the ratio 340/380 nm wavelengths (R340/380, ordinate). The initial recording is the basal $[Ca^{2+}]_c$ (arrows) that was followed by an acetylcholine challenge (100 μM), as shown by the horizontal bottom bar. B, pooled results from the number of experiments shown in parentheses. C, a prototype experiment to monitor the basal $[Ca^{2+}]_{er}$ as indicated by arrows (measured as the R340/380, ordinate) and the changes evoked by a mixture of 10 μM ryanodine, 2 μM thapsigargin and 20 mM ($C_{20}RT$) or 50 mM caffeine ($C_{50}RT$). D, pooled results of the basal $[Ca^{2+}]_{er}$ from normotensive and hypertensive rat slices, calculated from the initial part of the traces shown in panel C (arrows). E, example records from a prototype experiment to monitor the variations of mitochondrial $[Ca^{2+}]$ (measured as arbitrary fluorescence units, AFU, ordinate) in Rhod-2-loaded adrenal slices, under basal conditions (arrows) and after adding the protonophore FCCP at the indicated concentrations. F, pooled results on the basal $[Ca^{2+}]_m$ calculated from the initial part of the traces shown in panel E (arrows). Data in panels B, D and F are means ± S.E.M. of the number of experiments shown in parentheses. ***$P<0.001$ with respect to control rats.

3.2. $[Ca^{2+}]_c$ elevations elicited by acetylcholine or K^+ stimulation of Fura-2-loaded adrenal medulla slices

All experiments began with an initial 5-min perifusion resting period to adapt the slice to its new environment. Once the basal

fluorescence was stable, the normal Tyrode solution containing 1.8 mM Ca^{2+} was switched to another containing 100 μM acetylcholine that bathed the slice during 80 s. Fig. 2A shows fluorescence example images from a NWR in basal conditions, after challenging with acetylcholine, and the final maximal fluorescence elicited by digitonin (panels 1, 2, and 3, respectively). The bottom panels show the fluorescence signal produced in a SHR slice, that was more intense under the three experimental conditions i.e., basal, acetylcholine, and digitonin (panels 4, 5 and 6), in comparison with the NWR slice.

Fig 2B shows representative records of the time courses of $[Ca^{2+}]_c$ elevations (measured as normalized fluorescence ratios at 340/380 wavelenghts) elicited by acetylcholine in a NWR slice and a SHR slice. The responses had two phases namely, an initial transient peak that was followed by a decay phase to reach a plateau. The amplitude of the peak and the plateau were measured as indicated by double head arrows. Although the two responses were qualitatively similar, they however exhibited clear quantitative differences, as shown in panels C and D of Fig. 2. For instance, the peak $[Ca^{2+}]_c$ was 59.7% higher in SHR and the plateau 101.4% greater, as compared with NWR. We also analyze the time constants for activation (τ_a) and inactivation (τ_i) of the $[Ca^{2+}]_c$ transients. So, τ_a were 25 s and 20 s and τ_i were 24.8 s and 34.6 s, respectively in NWRs and SHRs. Hence, τ_a was 20% lower in SHR and τ_i was 39% higher in SHRs, as compared with NWRs (Fig. 2D).

A picture similar to that for acetylcholine was found in slices stimulated with a Tyrode solution containing 70 mM K^+ (low Na^+). In the representative fluorescence micrographs shown in Fig. 3A, fluorescence intensities were substantially higher in SHR slices (compare panels 1, 2 and 3 with panels 4, 5 and 6), under the three experimental conditions (basal, K^+, and digitonin). The time courses of the changes in fluorescence elicited by K^+ are shown in Fig. 3B; the $[Ca^{2+}]_c$ peak was higher and sharper in SHR, compared with NWR. Again, the $[Ca^{2+}]_c$ declined to a plateau in both preparations. The bar graph of Fig. 3C represents averaged results obtained in 15 slices from NWRs and SHRs challenged with K^+. The fluorescence peak was 44.4% higher and the plateau 88.3% higher in SHRs, compared with NWRs. As in the case of acetylcholine, the $[Ca^{2+}]_c$ time course curves showed differences in their time constant for activation and inactivation. Thus, τ_a were 19.6 s and 14.3 s and the τ_i were 28.2 s and 45 s, respectively in NWRs and SHRs. So, the τ_a was 27% lower in SHR and τ_i was 59% higher in SHR, as compared with NWR slices (Fig. 3D).

In summary, chromaffin cells from SHR adrenal slices generated a substantially greater peak and plateau elevations of $[Ca^{2+}]_c$ as compared with the responses of chromaffin cells from NWR slices. Furthermore, activation of Ca^{2+} transients were faster and inactivation slower in SHR slices.

3.3. $[Ca^{2+}]_c$ elevations elicited by CRT, FCCP, and combined CRT plus FCCP (CRT/FCCP) in Fura-2-loaded adrenal medulla slices

So far, we have explored the $[Ca^{2+}]_c$ elevations produced by enhanced Ca^{2+} entry upon depolarizing stimuli. We wanted next to study the $[Ca^{2+}]_c$ increases produced by Ca^{2+} release from organelles. To induce Ca^{2+} release from the endoplasmic reticulum we used CRT, a mixture of 20 mM caffeine, 10 μM ryanodine, and 2 μM thapsigargin

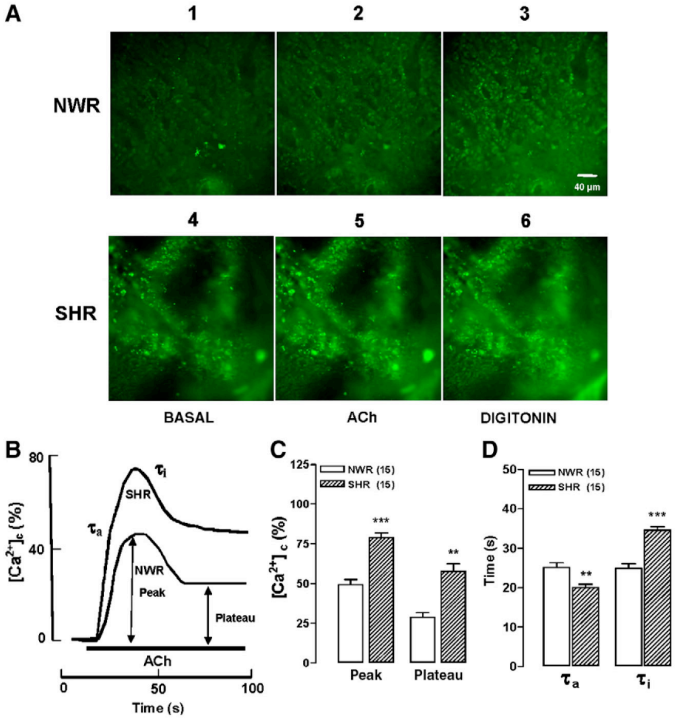

Fig. 2. Acetylcholine-induced $[Ca^{2+}]_c$ elevations were faster and higher in adrenal slices of SHRs, as compared with NWRs. Slices were loaded with Fura-2.AM and were continuously perifused with an oxygenated (95% O_2/5% CO_2) Tyrode solution containing 1.8 mM Ca^{2+}. They were challenged with 100 μM acetylcholine for 80 s. A, digital images showing the Fura-2 fluorescence under basal, acetylcholine, or digitonin treatment conditions (panels 1, 2 and 3 for NWR and 4, 5 and 6 for SHR slices). B, typical records representing the fluorescence of Fura-2 (R340/380, normalized as % of maximal fluorescence) in NWR and SHR slices stimulated with acetylcholine (bottom horizontal bar). C, averaged results from 15 NWRs and SHRs slices. D, time constants for activation (τ_a) and inactivation to a plateau (τ_i) of the $[Ca^{2+}]_c$ transients. The parameters plotted in C and D were calculated from experiments of the type shown in panel B. Data are means ± S.E.M., showing differences for SHR (*$P<0.05$ and ***$P<0.001$) compared with NWRs.

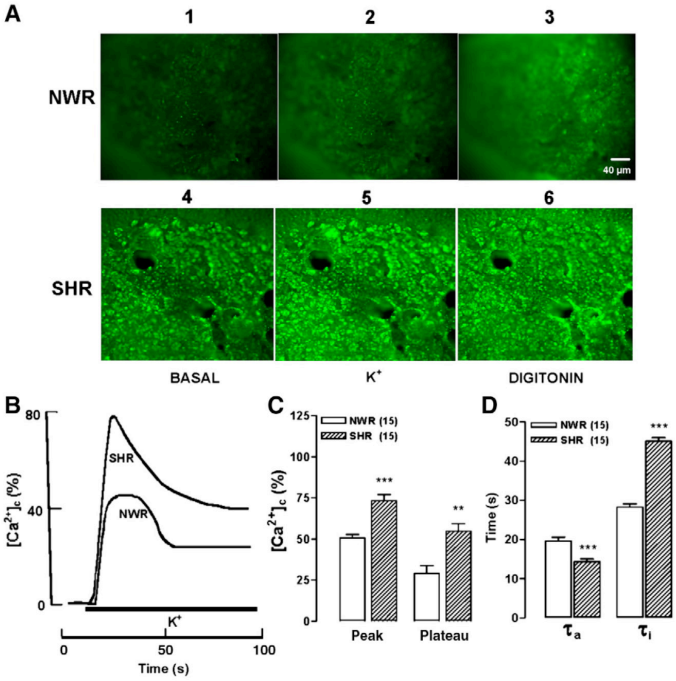

Fig. 3. Stimulation with a high-K^+ solution produced $[Ca^{2+}]_c$ elevations that were faster and higher in adrenal slices of SHRs, as compared with NWRs. After a 5-min pre-equilibration period, slices were challenged with 70 mM K^+ during 80 s. A, digital images showing the Fura-2 fluorescence under basal, K^+, or digitonin treatment conditions for NWRs (panels 1, 2 and 3) and SHRs (panels 4, 5 and 6). B, typical records representing the time course of Fura-2 fluorescence of (R340/380, normalized as % of maximal fluorescence) in NWR and SHR slices stimulated with K^+ during the time period shown by the bottom horizontal bar. C, averaged results from 15 NWR and 15 SHR slices. D, time constants for activation (τ_a) and inactivation to a plateau (τ_i) of the $[Ca^{2+}]_c$ transients. The parameters plotted in C and D were calculated in each individual slice, from traces similar to those shown in panel B. Data are means ± S.E.M. for SHR, showing differences (**$P<0.01$, ***$P<0.001$) with respect to NWRs.

(Cuchillo-Ibanez et al., 2002). To release the Ca^{2+} accumulated in the mitochondrial matrix, we used 5 µM of the protonophore FCCP (Montero et al., 2000). Except for the stimuli, the protocol was similar to that used for acetylcholine and K^+ in previous sections.

Fig. 4 shows representative records of Fura-2-loaded slices, one from a NWR and the other from a SHR. In contrast to the responses elicited by acetylcholine or K^+ (Figs. 1–3), here the $[Ca^{2+}]_c$ elevations evoked by CRT (Fig. 4A) had not a biphasic pattern, but directly rose to a plateau with little (in the NWR trace) or no decline at all (in the SHR trace). The bar graph of Fig. 4B shows the averaged results of 15 slices from each animal type; the $[Ca^{2+}]_c$ plateau was 32.3% higher in SHRs, as compared with NWRs.

Fig. 4C and D shows the results of experiments performed with FCCP. The time courses of the $[Ca^{2+}]_c$ elevations were similar to those found with CRT, i.e., a direct rise to a stable plateau of the $[Ca^{2+}]_c$ signal. Once more, the plateau amplitude was higher in SHR (52.6%), as compared with NWR slices. Finally, experiments were done to induce the simultaneous release of Ca^{2+} from the endoplasmic reticulum and mitochondria. Combined CRT/FCCP produced rapid $[Ca^{2+}]_c$ elevations to a stable plateau (Fig. 4E). The plateau amplitude of SHRs was 70.6% higher than in NWRs (Fig. 4F). Finally, we analyzed the τ_a for the rising phase of $[Ca^{2+}]_c$ transients. The τ_a with CRT were not significantly different in NWR (18.3 s) and SHR (19.5 s). However, the τ_a with FCCP were 18% faster in SHR (17.8 s) than in NWR (21.7 s). In contrast, the τ_a with CRT/FCCP were 19% slower in SHR (23.5 s) than in NWR (19.7 s) (Fig. 4G).

In summary, stimulation of Ca^{2+} release from the endoplasmic reticulum store or mitochondria, elicited greater elevations of the $[Ca^{2+}]_c$ in SHRs, as compared with NWRs. Such elevations were higher and faster when Ca^{2+} was released from mitochondria, as compared with the endoplasmic reticulum.

3.4. Direct measurement of Ca^{2+} release from the endoplasmic reticulum triggered by CRT, from Fura-FF-loaded slices of NWRs and SHRs

It has been shown that the $[Ca^{2+}]_c$ elevations elicited by CRT were due to Ca^{2+} release from the endoplasmic reticulum into the cytosol of chromaffin cells (Alonso et al., 1999; Cuchillo-Ibanez et al., 2002). We therefore decided to measure the rates of $[Ca^{2+}]_{er}$ release elicited by CRT using Fura-FF, a probe that specifically monitors the changes of Ca^{2+} occurring at the endoplasmic reticulum. We used two caffeine concentrations in the CRT mixture i.e., 20 mM (C_{20}RT) and 50 mM (C_{50}RT), that were sequentially added to the same slice.

Fig. 5A shows representative fluorescence images obtained from a Fura-FF-loaded NWR slice (panels 1–4) and a SHR slice (panels 5–8). The time courses of $[Ca^{2+}]_{er}$ decreases were quite fast in both NWR (Fig. 5B) and SHR slices (Fig. 5C). The extent of $[Ca^{2+}]_{er}$ decrease elicited by C_{20}RT reached about 40% in both NWRs and SHRs; upon use of the solution C_{50}RT, depletion accounted for around 65% with no differences between normotensive and hypertensive animals (Fig. 5D).

The time constants for the rates of decay of $[Ca^{2+}]_{er}$, in slices challenged with C_{20}RT (τ_1) and C_{50}RT (τ_2) are shown in Fig. 5E. Decay τ_1 were 11.7 s for NWRs and 12.8 s for SHRs; τ_2 were 11.9 s for NWRs and 13.4 s for SHRs. Thus, no differences were found as far as the rate and extent of Ca^{2+} mobilization from the ER of NWRs and SHRs.

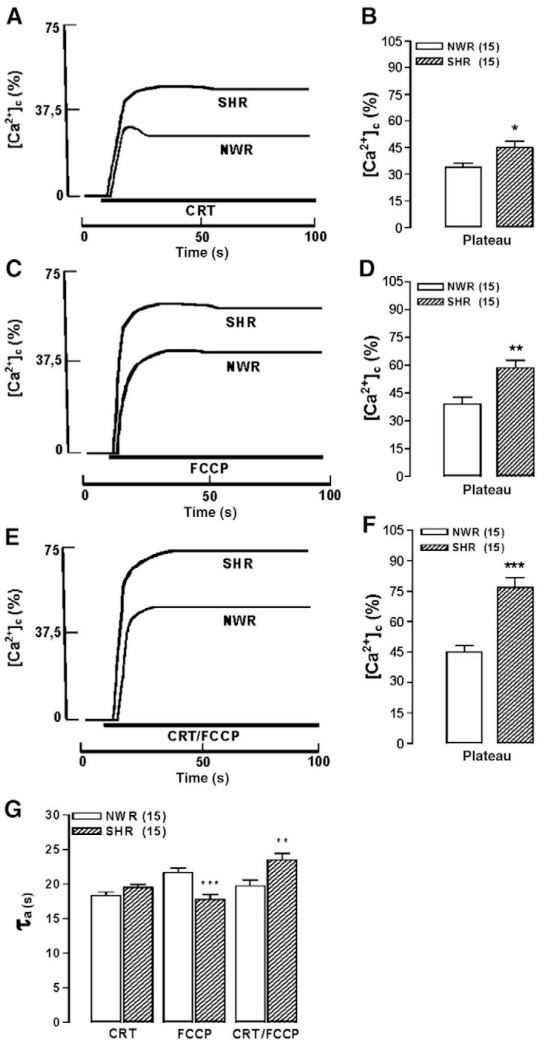

Fig. 4. Elevations of the $[Ca^{2+}]_c$ elicited by CRT, FCCP, or combined CRT plus FCCP (CRT/FCCP) in Fura-2-loaded slices from NWRs and SHRs. After a 5 min time period of pre-equilibration, slices were perifused with a Tyrode solution containing a mixture of 20 mM caffeine, 10 μM ryanodine, and 2 μM thapsigargin (CRT) or 5 μM FCCP, or both treatments in combination (CRT/FCCP). A, C and E are example traces of the $[Ca^{2+}]_c$ elevations (normalized as % of maximal fluorescence) elicited by the treatments indicated by the bottom bar below each trace. B, D and F are quantitative data on the plateau amplitude in slices from 15 NWR or SHR adrenals, corresponding to experiments done with protocols as shown in the respective left-hand slices. G, time constants for activation (τ_a) of the $[Ca^{2+}]_c$ elevations, calculated for the 15 slices used for each protocol, as shown in panels A, C, and E. Data in the bar graphs are means ± S.E.M. of 15 slices for each experimental condition showing the differences between NWRs and SHRs. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with NWRs (panels B, D, F and G).

3.5. Monitoring of mitochondrial Ca^{2+} release triggered by FCCP from Rhod-2-loaded slices of NWRs and SHRs

The protonophore FCCP dissipates the H^+ gradient and causes mitochondrial depolarization. This leads to interruption of Ca^{2+} uptake and the release into the cytosol of mitochondrial Ca^{2+} (Duchen, 2000), thus explaining the $[Ca^{2+}]_c$ elevation produced by FCCP in the experiments shown in Fig. 4C,D. A more direct measure of $[Ca^{2+}]_m$ changes elicited by FCCP was attempted using the mitochondrial calcium probe Rhod-2. Fig. 6A shows fluorescence images taken from a NWR slice (panels 1–4) or a SHR slice (panels 5–8). The basal fluorescence was considerably higher in SHR, as compared with NWR (panels 1 and 5). This difference was more than double, as indicated in the bar graph of Fig. 1F on pooled results from 10 preparations. Also, fluorescence was more intense in SHR slices treated with 5 and 10 μM FCCP, compared with NWR slices.

Fig. 6B displays an original trace of the $[Ca^{2+}]_m$ changes elicited by two concentrations of FCCP added sequentially to the perifusion solution. Upon addition of 5 μM FCCP, $[Ca^{2+}]_m$ decayed quickly to reach a new steady state level at 30% of the initial. Then, the subsequent addition of 10 μM FCCP produced further Ca^{2+} loss that decayed until 52% of the initial value. The effects of FCCP were faster and higher in the representative records on $[Ca^{2+}]_m$ obtained from a SHR slice (Fig. 6C). At 5 μM, FCCP caused a 52% Ca^{2+} depletion and at 10 μM the $[Ca^{2+}]_m$ decayed by almost 90%. The decay was higher in SHR, as compared with NWR slices. Fig. 6D summarizes the effects of FCCP on $[Ca^{2+}]_m$, which caused a 62.5% (FCCP 5 μM) and 40% (FCCP 10 μM) greater Ca^{2+} release in SHR, as compared with NWR slices. The Ca^{2+} decay time constants were 69% smaller at 5 μM FCCP and 60% smaller at 10 μM FCCP in SHR, compared with NWR slices. In summary, FCCP caused a greater and faster Ca^{2+} release from mitochondria of SHR chromaffin cells, compared with NWRs.

3.6. Calcium movements between the endoplasmic reticulum and mitochondria, in NWR and SHR slices challenged with FCCP or $C_{50}RT$

The mitochondrial Ca^{2+} uniporter can see the $[Ca^{2+}]_c$ elevation produced by the release of Ca^{2+} from the endoplasmic reticulum in the presence of caffeine (Montero et al., 2000). It was therefore of interest to study if the $[Ca^{2+}]_c$ elevations produced by CRT and FCCP were seen by mitochondria and the endoplasmic reticulum respectively, and whether this Ca^{2+} movements between the two organelles were distinct in NWR and SHR adrenal slices.

In the experiment of Fig. 7A, Fura-FF-loaded slices were exposed to FCCP, to test whether the Ca^{2+} released from mitochondria was taken up by the endoplasmic reticulum. In the NWR slice, the $[Ca^{2+}]_{er}$ was quickly increased upon FCCP treatment; the Ca^{2+} uptake curve reached a plateau after a couple of minutes. Addition of $C_{20}RT$ and $C_{50}RT$ on top of such plateau caused a prompt decay of Ca^{2+}, indicating that Ca^{2+} was indeed being released from the endoplasmic reticulum. Ca^{2+} released from mitochondria was taken up by the endoplasmic reticulum to a greater extent in SHR slices (about 40% more) compared with NWR slices (Fig. 7B). The time constant for endoplasmic reticulum Ca^{2+} uptake was 42% slower in SHRs; The τ_1 for Ca^{2+} decay elicited by $C_{20}RT$ was 8 s in NWR and 6 s in SHR, 25% faster in SHRs; and τ_2 for $C_{50}RT$ was 8.5 s in NWR and 6 s in SHR, 30% faster in SHR (Fig. 7C).

On the other hand, whether mitochondria were capable of taking up the Ca^{2+} released from the endoplasmic reticulum store was investigated in adrenal slices of NWRs and SHRs, loaded with Rhod-2. In the experiments of Fig. 7D, the Ca^{2+} being released from the endoplasmic reticulum into the cytosol by $C_{50}RT$ treatment was quickly taken up by mitochondria. The $[Ca^{2+}]_m$ reached a stable plateau in 42 s and 61% of maximum fluorescence in NWR, and 45 s and 65% of maximum fluorescence in SHR.

The fact that $[Ca^{2+}]_m$ quickly decayed upon FCCP treatment suggests that Ca^{2+} released to the cytosol from the endoplasmic reticulum was certainly taken up by mitochondria. No differences were observed on the maximum $[Ca^{2+}]_m$ reached in NWR and SHR slices (Fig. 7E). Furthermore, the time constants for mitochondrial Ca^{2+} release were 17 s in NWR and 12 s in SHR; therefore, they were 29% faster in SHR, compared with NWR slices (Fig. 7F).

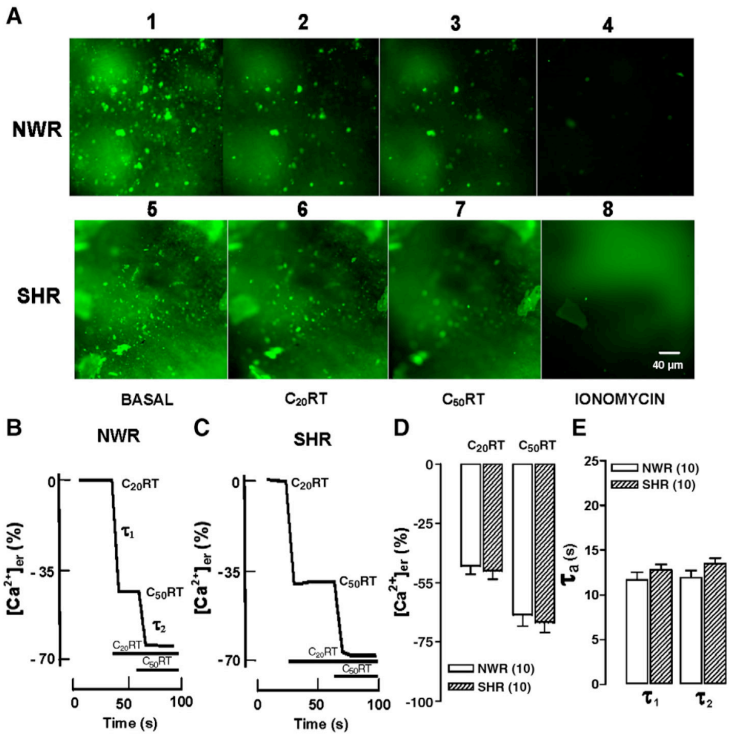

Fig. 5. Rapid depletion by CRT of the $[Ca^{2+}]_{er}$, as shown in slices from NWRs and SHRs loaded with the calcium fluorescent probe Fura-FF. After a 5-min pre-equilibration period, the slices were perifused with a Tyrode solution containing 20 mM caffeine, 10 μM ryanodine, and 2 μM thapsigargin ($C_{20}RT$) or subsequently with a second solution containing 50 mM caffeine, 10 μM ryanodine, and 2 μM thapsigargin ($C_{50}RT$). A, panels 1–4 and 5–8 show the fluorescence images taken just before addition of $C_{20}RT$ (basal), and upon addition of $C_{50}RT$, or ionomycin, respectively for NWR and SHR slices. B and C, time course of the $[Ca^{2+}]_{er}$ depletion in a NWR (panel B) or SHR slice (panel C), elicited by the cumulative additions of $C_{20}RT$ and $C_{50}RT$. D, pooled data on the extent of $[Ca^{2+}]_{er}$ reduction elicited by $C_{20}RT$ and $C_{50}RT$ in NWR and SHR slices. E, time constants for the rates of $[Ca^{2+}]_{er}$ decay upon treatment with $C_{20}RT$ (τ_1) or $C_{50}RT$ (τ_2). Data in panels D and E are means ± S.E.M. of 10 experiments.

In summary, the FCCP-elicited Ca^{2+} release from mitochondria into the cytosol was promptly taken up by the endoplasmic reticulum. Greater Ca^{2+} uptake in SHR slices indicates a larger capacity of the endoplasmic reticulum of SHR to accumulate the Ca^{2+} released from mitochondria. Conversely, Ca^{2+} released from the endoplasmic reticulum into the cytosol was quickly taken up by mitochondria; this Ca^{2+} uptake was similar in NWR and SHR slices.

4. Discussion

In this study we have found notable differences in the mechanisms that control Ca^{2+} homeostasis in adrenal medullary chromaffin cells of normotensive and hypertensive rats. As in smooth muscle cells (Jelicks and Gupta, 1990; Sugiyama et al., 1986), we found here that the $[Ca^{2+}]_c$ in resting chromaffin cells of adrenal slices was considerably higher in SHRs, compared with NWRs. Mitochondria of rat chromaffin cells take up Ca^{2+} from the cytosol, particularly when such $[Ca^{2+}]_c$ is elevated (Babcock et al., 1997); it is therefore plausible that higher $[Ca^{2+}]_m$ of SHRs can be secondary to enhanced $[Ca^{2+}]_c$ seen in resting chromaffin cells of these hypertensive animals.

The $[Ca^{2+}]_c$ transients elicited by chromaffin cell depolarization with acetylcholine or K^+ were also higher in SHRs, compared with NWRs. These $[Ca^{2+}]_c$ elevations are due to enhanced Ca^{2+} entry, as first shown by Douglas and Poisner (1961). Consequently, higher $[Ca^{2+}]_c$ elevations in SHRs could be due to higher expression of a given subtype (i.e. L, N and P/Q) of voltage-dependent Ca^{2+} channels described in rat chromaffin cells (Gandia et al., 1995). Differences in the kinetics of those channels, that undergo voltage- (Villarroya et al., 1999) and Ca^{2+}-dependent inactivation (Hernández-Guijo et al., 2001) in chromaffin cells, could also explain the higher $[Ca^{2+}]_c$ elevations seen in SHRs, with respect to NWRs, as well as their biphasic pattern. We are now initiating a study in voltage-clamped NWR and SHR chromaffin cells, to investigate the components of whole-cell inward Ca^{2+} currents as well as their activation, inactivation, and deactivation kinetics.

Higher $[Ca^{2+}]_c$ elevations in SHRs chromaffin cells were also seen upon Ca^{2+} mobilization from the endoplasmic reticulum or mitochondria. Nomura and Asano (2002) found an increased Ca^{2+} buffering capacity of sarcoplasmic reticulum in small mesenteric arteries from SHRs. We also found a greater Ca^{2+} uptake into the endoplasmic reticulum, upon mobilization by FCCP of mitochondrial Ca^{2+}, implying a larger Ca^{2+} store in SHRs, compared with NWRs. This was supported by greater $[Ca^{2+}]_c$ elevations elicited by CRT (Fig. 4A,B) but not by similar $[Ca^{2+}]_{er}$ depletion in both NWRs and SHRs (Fig. 5D).

An alteration of the ability of mitochondria to take up Ca^{2+} from the cytosol, and to release it back to the cytosol, the so-called mitochondrial Ca^{2+} cycling (mCC) (Duchen, 2000; Garcia et al., 2006) has been implicated in the pathogenesis of hypertension. The following findings support this idea: (1) Arab et al. (1990) observed an augmentation of mitochondrial Ca^{2+} uptake through the uniporter in smooth muscle cells during hypertension; (2) this may lead to enhanced $[Ca^{2+}]_m$ (Fig. 1E,F of this study) that could augment the opening probability of the mitochondrial permeability transition pore

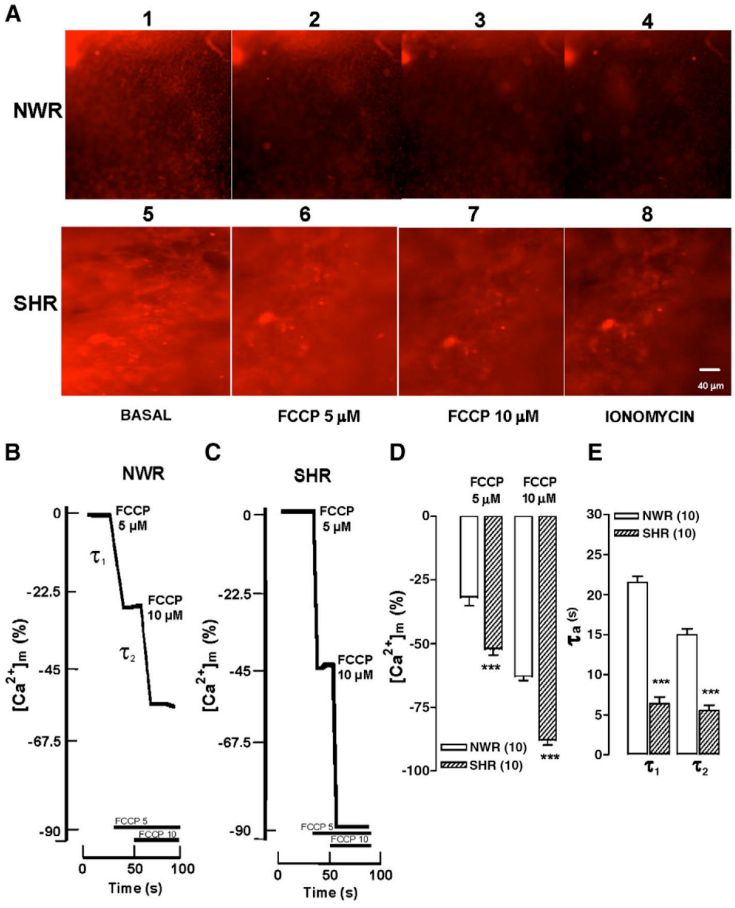

Fig. 6. FCCP causes a greater and faster Ca^{2+} release from mitochondria of SHR adrenal slices, as compared with NWR slices. Slices loaded with the mitochondrial Ca^{2+} fluorescence probe Rhod-2 were continuously perifused with an oxygenated Tyrode solution containing 1.8 mM Ca^{2+}. After a 5-min equilibration period, they were exposed to the protonophore FCCP at the indicated concentrations. **A**, panels 1–4 and 5–8 show the fluorescence images taken just before (basal) or at the end of FCCP (5 or 10 µM) or ionomycin (1 µM) additions. B and C, traces showing the time course of the $[Ca^{2+}]_m$ decay, obtained from slices of NWR (panel B) and SHR adrenals (panel C). D, pooled data on the extent of $[Ca^{2+}]_m$ depletion elicited by the two concentrations of FCCP. E, time constants for the rates of $[Ca^{2+}]_m$ decay upon FCCP treatment. Data in panels D and E are means ± S.E.M. of 10 experiments. ***$P<0.001$, SHRs compared with NWRs.

(mPTP) with an increase of free radical production and reduction of NO-elicited vasodilatation (Postnov Iu, 2004); (3) in the brainstem of SHRs where centers for blood pressure regulation are located, Lopez-Campistrous et al. (2008) found a decrease of ATP synthesis and an enhanced production of free radicals; and (4) the production of ATP is impaired in liver and brain mitochondria from adult SHRs (Budnikov et al., 2002; Doroshchuk et al., 2004). Impaired ATP synthesis may lead to a decreased activity of ATP-dependent plasmalemmal Ca^{2+}-ATPase and the sarco-endoplasmic reticulum ATPase (SERCA); this could explain the higher $[Ca^{2+}]_c$ and the enhanced mCC that becomes a vicious circle eventually leading to opening of the mPTP, the production of free radicals, and cell damage.

Concerning the profile of the $[Ca^{2+}]_c$ elevation curves evoked by stimuli that trigger Ca^{2+} entry (i.e. acetylcholine, K^+) or Ca^{2+} release from the endoplasmic reticulum and mitochondria (i.e. CRT, FCCP) we found two major differences; (1) responses with phasic and tonic components upon stimulation with acetylcholine or K^+; and (2) responses with only a tonic component upon stimulation with CRT or FCCP. The simplest explanation for these differences may be linked to Ca^{2+} and voltage inactivation of high-voltage activated Ca^{2+} channels (Hernández-Guijo et al., 2001) that open during chromaffin cell depolarization with acetylcholine or K^+ (Douglas and Rubin, 1961, 1963). On the other hand, Ca^{2+}-induced Ca^{2+} release (CICR), shown to be activated by Ca^{2+} entry through Ca^{2+} channels of chromaffin cells (Alonso et al., 1999) could contribute to the tonic component of the $[Ca^{2+}]_c$ elevation triggered by acetylcholine (Fig. 2B) or K^+ (Fig. 3B). It is interesting that the time constant for the decay of the peak $[Ca^{2+}]_c$ transient was slower in SHR slices stimulated with acetylcholine (Fig. 2D) and K^+ (Fig. 3D). This suggests that Ca^{2+} channels may inactivate more slowly in SHRs. This could indicate some difference in the voltage-sensor located in the structure of calcium channels in SHRs.

A functional triad formed by Ca^{2+} channels, the endoplasmic reticulum, and mitochondria has been suggested to shape $[Ca^{2+}]_c$ transients and catecholamine release responses upon chromaffin cell depolarization (Garcia et al., 2006). On the basis of measurements of

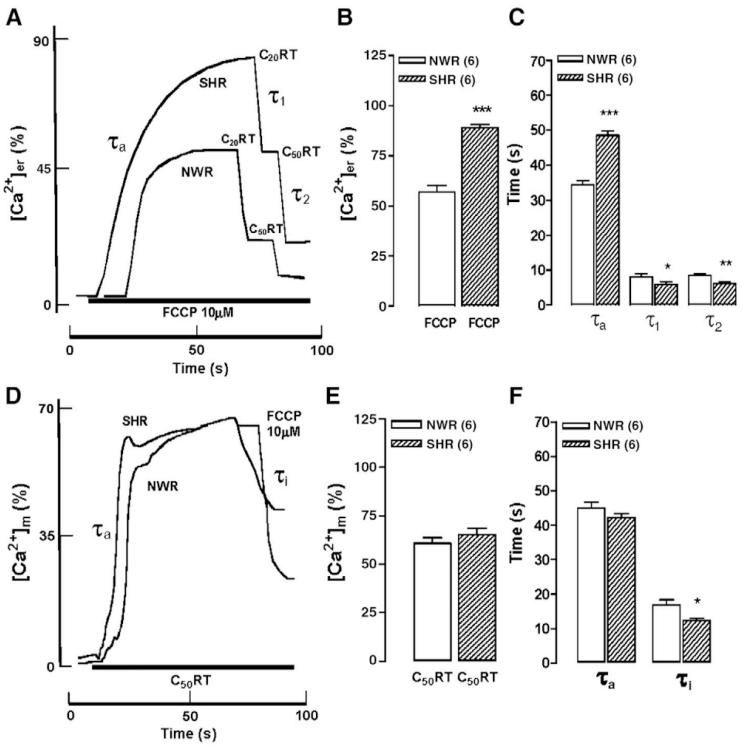

Fig. 7. Ca^{2+} movements between the endoplasmic reticulum and mitochondria, in NWR and SHR adrenal slices. These experiments were performed in Fura-FF-loaded slices to monitor the uptake into the endoplasmic reticulum of Ca^{2+} coming from mitochondria (panels A, B and C), or in Rhod-2-loaded slices, to see the uptake into mitochondria of Ca^{2+} coming from the endoplasmic reticulum (panels D, E and F). A, example traces of an experiment performed in Fura-FF-loaded slices. $[Ca^{2+}]_{er}$ is expressed as % of the maximum fluorescence (ordinate). FCCP was perifused as indicated by the bottom horizontal bar; $C_{20}RT$ and $C_{50}RT$ were given as indicated. B, pooled results on the maximum $[Ca^{2+}]_{er}$ reached in slices treated with FCCP, normalized as % of the maximum fluorescence (ordinate). C, time constants for the activation (τ_a rate of endoplasmic reticulum Ca^{2+} uptake upon FCCP addition) and $[Ca^{2+}]_{er}$ decay upon addition of $C_{20}RT$ (τ_1) of $C_{50}RT$ (τ_2). D, example traces from an experiment performed in Rhod-2 loaded slices. $[Ca^{2+}]_m$ is expressed as % of maximum fluorescence (ordinate). $C_{50}RT$ was perifused as indicated by the bottom horizontal bar; FCCP was given as indicated. E, pooled data on the maximum $[Ca^{2+}]_m$ reached in slices treated with $C_{50}RT$, normalized as % of the maximum fluorescence (ordinate). F, time constants for activation (τ_a, rate of mitochondrial Ca^{2+} uptake upon $C_{50}RT$ addition) and $[Ca^{2+}]_m$ decay upon addition of FCCP (τ_i). Data in panels B, C, E, and F are means ± S.E.M. of 6 experiments. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, SHRs compared with NWRs.

single-cell quantal catecholamine release, we recently speculated that this functional triad was not altered in SHR chromaffin cells (Miranda-Ferreira et al., 2009). However, in the light of our present experiments on Ca^{2+} handling by chromaffin cells, it seems that such earlier conclusion should be reconsidered. Nevertheless, it is not easy to correlate Ca^{2+} changes occurring in the cytosol and organelles during cell stimulation, with pre-exocytotic phases and the last steps of exocytosis. For instance, the higher basal $[Ca^{2+}]_c$ in SHRs (Fig. 1A,B, this study) could increase vesicle movement and cause a greater loading of a docked vesicle pool (von Ruden and Neher, 1993); this could explain the longer secretory responses triggered by acetylcholine and K^+ in SHR, compared with NWR chromaffin cells (as in Figs. 1 and 2 in Miranda-Ferreira et al., 2009). Also, the greater $[Ca^{2+}]_c$ elevation elicited by CRT and FCCP in SHRs (Fig. 4, this study) could explain the longer secretory responses elicited by CRT and FCCP in SHR chromaffin cells (as in Figs. 3 and 4 in Miranda-Ferreira et al., 2009). Furthermore, the higher basal $[Ca^{2+}]_m$ (Fig. 1E,F, this study) and the greater mitochondrial Ca^{2+} release upon FCCP treatment (Fig. 6, this study) suggest a more relevant role of mitochondrial Ca^{2+} uptake and release in controlling $[Ca^{2+}]_c$ and exocytosis in SHR chromaffin cells. This may explain that FCCP pretreatment, that suppresses mitochondrial Ca^{2+} cycling, caused a more drastic augmentation of the secretory responses triggered by acetylcholine or K^+ in SHR, as compared with NWR chromaffin cells (as in Fig. 6 in Miranda-Ferreira et al., 2009). Finally, depletion by CRT pretreatment of endoplasmic reticulum Ca^{2+} also caused a potentiation of acetylcholine and K^+ secretory responses that was greater in SHRs, compared with NWRs (as in Fig. 5 in Miranda-Ferreira et al., 2009). Although indirectly, this finding suggests that during cell activation with acetylcholine or K^+, endoplasmic reticulum Ca^{2+} uptake via SERCA into the Ca^{2+} store is substantially greater in SHRs. This supports the finding of Levitsky et al. (1993) who found a greater SERCA expression in SHRs; however, it disagrees with other studies showing a smaller expression (Arata et al., 1999; Limas and Cohn, 1977), or no change in SERCA expression in SHR, with respect to NWR ER (Le Jemtel et al., 1993).

The possible relevance of our results for understanding the pathophysiology of human hypertension and the identification of novel therapeutic targets deserves a last comment. Numerous studies support the notion that pre- and postsynaptic sympathetic dysfunctions are involved in the pathophysiology of human or animal primary hypertension (de Champlain et al., 1976; Tsuda and Masuyama, 1991). Studies on presynaptic mechanisms have been performed in SHRs, showing that norepinephrine release is increased in different tissues rich in sympathetic nerve endings (Donohue et al., 1988). Furthermore, plasma levels of norepinephrine and epinephrine are augmented in

SHRs (Grobecker et al., 1975; Iriuchijima, 1973; Pak, 1981), as happens to be the case in humans suffering essential hypertension (Goldstein et al., 1983). This may be due to elevated sympathetic nerve activity (Anderson et al., 1989) leading to enhanced catecholamine release from sympathetic nerves and chromaffin cells (Alonso et al., 1999; Caricati-Neto et al., 1992; Donohue et al., 1988; Lim et al., 2002; Miranda-Ferreira et al., 2008, 2009; Tsuda and Masuyama, 1991). The present study suggests that a dysfunction of mitochondrial Ca^{2+} cycling may be the basis for enhanced Ca^{2+} availability to the secretory machinery; this could explain the augmented secretory responses in sympathetic neurons and chromaffin cells, that can be associated to the greater vasoconstrictor responses and smaller vasodilatory responses underlying the pathogenesis of hypertension (Arii et al., 1999; Fellner and Arendshorst, 2002; Nomura et al., 1997; Sugiyama et al., 1986; van Breemen et al., 1987). If true, the pharmacological regulation of mitochondrial Ca^{2+} cycling could become a new target for the development of new antihypertensive drugs; this pharmacological stabilisation of mitochondrial Ca^{2+} cycling could afford protection to sympathoadrenal and cardiovascular tissues that are exposed to oxidative stress in hypertensive states. In fact, antioxidants produce a fall in blood pressure in a transgenic mouse model of hypertension (Tornavaca et al., 2009).

In conclusion, we have found here that the handling of Ca^{2+} by chromaffin cells in their natural adrenal slice environment considerably differ in normotensive and hypertensive rats. Basal Ca^{2+} concentrations in the cytosol and mitochondria as well as the $[Ca^{2+}]_c$ elevations triggered by depolarization or mitochondrial poisoning with FCCP are greater in SHR chromaffin cells, compared with adrenal slices from normotensive rats. Overall, these changes of Ca^{2+} homeostatic mechanisms could explain the greater quantal catecholamine release responses observed in SHR, as compared with NWR chromaffin cells (Miranda-Ferreira et al., 2008, 2009).

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Lower density of L-type and higher density of P/Q-type of calcium channels in chromaffin cells of hypertensive, compared with normotensive rats

Ricardo de Pascual [1,a,b], Regiane Miranda-Ferreira [d,1], Kleber M. Galvão [d], Claudina Lameu [e], Henning Ulrich [e], Soraya S. Smaili [d], Aron Jurkiewicz [d], Antonio G. García [a,b,c], Luis Gandía [a,b,*]

[a] Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, Spain
[b] Departamento de Farmacología y Terapéutica, Facultad de Medicina, Universidad Autónoma de Madrid, Spain
[c] Servicio de Farmacología Clínica, Instituto de Investigación Sanitaria, Hospital Universitario de la Princesa, Universidad Autónoma de Madrid, Spain
[d] Departamento de Farmacologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil
[e] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Brazil

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ABSTRACT

Enhanced activity of the sympatho-adrenal axis and augmented circulating catecholamines has been implicated in the development of hypertension. Release of catecholamine from stimulated adrenal medulla chromaffin cells has been shown to be higher and longer in spontaneously hypertensive rats (SHRs), compared with normotensive Wistar rats (NWRs). Whether differences in the functional expression of voltage-dependent calcium channels (VDCCs) of the L-, N-, or P/Q subtypes may contribute to such distinct secretory behaviour, is unknown. We therefore approached here this study in voltage-clamped NWR and SHR chromaffin cells, using 10 mM Ba^{2+} as charge carrier (I_{Ba}) and selective blockers of each channel type. We found that compared with NWR cells, SHR chromaffin cells exhibited the following differences: (1) 30% diminution of the I_{Ba} fraction carried by L channels; (2) a doubling of the I_{Ba} fraction carried by P/Q channels; (3) more visible current modulation by ATP that could be linked to a 10-fold higher mRNA levels for purinergic receptors of the P_{2Y2} subtype; and (3) a higher contribution of PQ channels to the transients of the cytosolic calcium concentrations ($[Ca^{2+}]_c$) generated by K^+, compared with L channels. These results may contribute to the better understanding of the greater calcium signalling and exocytotic responses of SHR compared with NWR chromaffin cells, found in three previous reports from our laboratories.

1. Introduction

In trying to understand the pathogenesis and hence the drug therapy of human hypertension, the genetic spontaneously hypertensive rat (SHR) has extensively been used as a relevant experimental model (Okamoto and Aoki, 1963; Pinto et al., 1998). Since long, enhanced activity of the sympatho-adrenal axis has been implicated as a central stage in the development of a hypertensive state (Anderson et al., 1989; de Champlain et al., 1999). There is clinical and experimental evidence in support of this hypothesis. For instance, borderline hypertensive patients have increased sympathetic nerve activity (Anderson et al., 1989) and enhanced plasma levels of noradrenaline and adrenaline (de Champlain et al., 1999; Goldstein, 1983). These enhanced levels of circulating catecholamines are also found in SHRs (Grobecker et al., 1975; Iriuchijima, 1973; Pak, 1981). The enhanced circulating catecholamine levels are likely due to augmented noradrenaline release from sympathetic nerves and adrenaline release from adrenal medulla chromaffin cells (Arnaiz et al., 1978). In this line is the observation that greater catecholamine release occurs in adrenal glands of SHRs, compared with normotensive rats (Lim et al., 2002).

For the last few years, our laboratory has been engaged in the study of calcium signalling and the amperometric analysis of the single-event kinetics of quantal release of catecholamines from chromaffin cells isolated from adrenal glands of normotensive Wistar rats (NRWs) and SHRs. We have found that SHR chromaffin cells stimulated with short pulses of acetylcholine or high K^+ (K^+) caused a higher production of individual spike secretory events and a drastic augmentation of the quantal catecholamine content of single secretory vesicles that exhibited faster fusion kinetics, compared with NWR chromaffin cells (Miranda-Ferreira et al., 2008). Secretory quantal release responses were also greater and of longer duration with respect to NWRs when challenging SHR chromaffin cells with a mixture of caffeine, ryanodine, and

* Corresponding author at: Instituto Teófilo Hernando, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 4. 28029 Madrid, Spain. Tel.: +34 91 497 31 20; fax: +34 91 497 37 40.
E-mail address: luis.gandia@uam.es (L. Gandía).
[1] Equal contributors.

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thapsigargin (CRT) to cause a rapid depletion of the entire calcium store of the endoplasmic reticulum (ER) (Cuchillo-Ibáñez et al., 2002), or the protonophore FCCP (carbonyl-cyanide p-trifluoromethoxy phenylhydrazone) to release Ca^{2+} from mitochondria and to prevent mitochondrial Ca^{2+} uptake during cell stimulation (Montero et al., 2000). We also found that K^+-elicited quantal release responses were markedly enhanced in chromaffin cells pretreated with CRT or FCCP (Miranda-Ferreira et al., 2009). We later on observed that the basal concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) and the basal concentration of Ca^{2+} in the mitochondrial matrix ($[Ca^{2+}]_m$) were substantially higher in chromaffin cells from SHRs, compared with NWRs. Furthermore, the $[Ca^{2+}]_c$ elevations triggered by Acetylcholine, K^+, CRT, and FCCP were also higher and exhibited faster activation and slower clearance in SHRs, with respect to NWRs (Miranda-Ferreira et al., 2010). These greater Ca^{2+} responses are in line with the longer and higher quantal catecholamine release responses in SHRs compared with NWRs, upon challenging their chromaffin cells with Acetylcholine or K^+ (Miranda-Ferreira et al., 2008), as well as with CRT or FCCP (Miranda-Ferreira et al., 2009).

The higher $[Ca^{2+}]_c$ transients elicited by chromaffin cell depolarisation with Acetylcholine or K^+ in SHRs, compared with NWRs, are shaped by a functional triad that includes the initial Ca^{2+} entry through plasmalemmal voltage-dependent calcium channels (VDCCs), and its subsequent redistribution into mitochondria and the ER (Garcia et al., 2006; Garcia et al., 2012). As described above, in trying to elucidate the role of Ca^{2+} in the greater secretory responses of chromaffin cells from SHRs with respect to NWRs, we previously explored two elements of this functional triad namely, the ER and the mitochondrion. Since the pioneering observation of William W. Douglas laboratory that Acetylcholine causes the release of catecholamine from the adrenal gland (Douglas and Rubin, 1961a) by enhanced Ca^{2+} entry (Douglas and Poisner, 1961), it has become clear that the contribution of VDCCs to the generation of $[Ca^{2+}]_c$ transients elicited by cell depolarisation is highly relevant. We now know that rat chromaffin cells express the subtypes of VDCCs L (α_{1D}, $Ca_v1.3$), N (α_{1B}, $Ca_v2.2$), and PQ (α_{1A}, $Ca_v2.1$) at different densities (Gandia et al., 1995). Furthermore, these VDCC subtypes undergo different voltage- (Villarroya et al., 1999) and Ca^{2+}-dependent inactivation (Hernández-Guijo et al., 2001) and they are modulated by endogenous ATP (Gandia et al., 1993) that is co-released with catecholamines during stimulation of chromaffin cells (Castillo et al., 1992).

The present study was planned to comparatively analyse the L, N, and PQ subcomponents of the whole-cell inward calcium channel currents, pharmacologically isolated with selective blockers in voltage-clamped chromaffin cells from NWRs and SHRs. Additionally, we also studied the regulation of such currents by ATP. Compared with NWRs, we found that SHR chromaffin cells present (1) a lower density of L-type VDCCs that is compensated by a higher density of PQ channels; (2) a more visible current modulation by ATP that could be linked to a 10-fold higher mRNA levels for purinergic receptors of the P_{2y2} subtype; and (3) a higher contribution of PQ channels, with respect to L channels, to the $[Ca^{2+}]_c$ transients generated by K^+. Attempts to explain these findings in the context of the more prolonged and sustained catecholamine release previously found to occur in SHRs with respect to NWRs, and in the frame of the functional triad, will be made in Section 4.

2. Materials and methods

2.1. Animals

Animals were manipulated according to the guidance of the Ethic Committee for Handling Research Animals of the Federal University of São Paulo (São Paulo, Brazil) and the Medical School of the Autonomous University of Madrid, Spain. Male 16-week-old Normotensive Wistar Rats (NWRs) and Spontaneously Hypertensive Rats (SHRs) weighing approximately 300 g were housed at 24 ± 2 °C, with 60% relative humidity, on a 12 h light/dark cycle. Animals were fed with a standard diet and water ad libitum.

2.2. Isolation and culture of rat adrenal medulla chromaffin cells from control and hypertensive rats

To prepare each cell batch we used 1–2 adult rats that were killed by cervical dislocation. The abdomen was opened, the adrenal glands exposed, and quickly removed and decapsulated and both adrenal medullae isolated under a stereoscope. They were placed in Ca^{2+}- and Mg^{2+}-free Locke buffer of the following composition (in mM): NaCl 154, KCl 3.6, $NaHCO_3$ 5.6, glucose 5.6, and Hepes 10 (pH 7.2) at room temperature. Tissues were collected under sterile conditions. Medullae digestion was achieved by incubating the pieces in 6 ml of Ca^{2+}/Mg^{2+}-free Locke buffer containing 6 mg collagenase, 12 mg bovine serum albumin, for 20 min at 37 °C; gentle shaking was applied at 5–10 min intervals by using a plastic Pasteur pipette. Collagenase was washed out of the cells with large volumes of Ca^{2+}/Mg^{2+}-free Locke buffer. The cell suspension was centrifuged at $120 \times g$ for 10 min. After washing 2 times, the cells were resuspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum containing 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin.

Cells were plated on circular glass coverslips, previously treated with 0.1 mg ml^{-1} of poly-D-lysine during 30 min, followed by a thorough washout with water. After 30 min, 1 ml DMEM was added to each well. Cells were then incubated at 37 °C in a water saturated, 5% CO_2 atmosphere; they were used within 1–2 days after plating.

2.3. Recording of Ca^{2+} channel currents

Membrane currents were measured using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Coverslips containing the cells were placed in an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused with a control Tyrode solution containing (in mM): NaCl 137, $MgCl_2$ 1, $CaCl_2$ 2, HEPES/NaOH 10, pH 7.4. For current recordings, 10 mM Ba^{2+} (instead of 2 mM Ca^{2+}) was used as a charge carrier. The cells were internally dialyzed with a solution containing (in mM): NaCl 10, CsCl 100, tetraethylammonium Cl 20, MgATP 5, Ethyleneglycol-bis-2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) 14, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES)/CsOH 20, Na.GTP 0.3, pH 7.2.

Whole-cell recordings were made with fire-polished glass electrodes (resistance 2–5 MΩ) mounted on the headstage of an EPC-9 patch-clamp amplifier, allowing cancellation of capacitative transients and compensation of series resistance. All recordings and data analysis were carried out with the software PULSE (HEKA Elektronik). Membrane currents were sampled at 10–20 KHz.

Unless indicated otherwise, the cells were clamped at −80 mV holding potential. Step depolarisation to 0 mV or +10 mV from this holding potential lasted 50 ms and were applied at 30-s interval to minimise current rundown (Fenwick et al., 1982).

External solutions were exchanged by a fast superfusion device consisting of a modified multi-barrelled pipette, the common outlet of which was positioned 50–100 μm from the cell. Control and test solutions were changed using miniature solenoid valves operated manually (The Lee Company, Westbrook, CT, U.S.A.). The flow rate (0.5–1 ml min^{-1}) was regulated

by gravity to achieve a complete replacement of cell surroundings in less than 1 s. All electrophysiological experiments were performed at room temperature (22–24 °C).

2.4. Preparation of adrenal medullary slices

Animals were sacrificed by decapitation and their adrenal glands were removed and immediately immersed in a Tyrode nutrient solution with the following composition (in mM): NaCl 137; KCl 5.4; $CaCl_2$ 1.8; $MgCl_2$ 1; $NaHCO_3$ 12; KH_2PO_4 0.36; glucose 11. The pH was adjusted at 7.3 with 95% O_2/5% CO_2. Adrenal glands were cleaned of fat, embedded in agarose (4%), and mounted in a vibratome chamber (1000 Plus Sectioning System, Campden Instruments, INC., U.S.A.) and cut in slices of 200 μm thickness. After incubation with Ca^{2+} indicators, the slices were mounted in a Leiden chamber of a fluorescence microscope (Nikon TE 300, Nikon, Osaka, Japan) coupled to a CCD camera (Quantix 512—Roper Scientific Inc., Princeton Instruments, NJ, U.S.A.) using the Spectralyser software (Paul Anderson, NJ, U.S.A.) to select a field containing chromaffin cells in the intact adrenal medullary tissue.

2.5. Cytosolic Ca^{2+} measurements in adrenal medullary slices

To study $[Ca^{2+}]_c$ changes, slices were incubated with 10 μM of the ratiometric dye fura-2.AM (Grynkiewicz et al., 1985) in a Tyrode solution containing 0.01% pluronic acid F-127 at room temperature for 45 min; this solution was oxygenated and protected from light. After incubation, slices were washed twice with Tyrode solution and mounted in a Leiden chamber with 1 ml of Tyrode solution and kept at 37 °C during the experiments. Basal $[Ca^{2+}]_c$ was measured during the first 10 images, which were taken at 5-s intervals.

Chromaffin cells from the adrenal medulla slices were challenged with KCl (70 mM) to stimulate Ca^{2+} influx through different VDCC subtypes. To study the relative participation of VDCCs on the calcium influx by K^+ stimulus, we initially incubated the adrenal slices with A) nifedipine (3 μM) to block L-type VDCCs; B) ω-conotoxin GVIA (1 μM) to block N-type VDCCs; or C) ω-agatoxin IVA (3 μM) to block P/Q-type VDCCs. After this incubation, we again challenged the slices using 70 mM K^+ in order to study the relative participation of each VDCC on the calcium influx stimulated by K^+. At the end of the experiment, calibration was done by adding 1 mM digitonin to permeabilise the cells and assure the maximal fura-2 fluorescence (Rmax). The minimal Ca^{2+} fluorescence (Rmin) is obtained under resting conditions of slices (basal).

2.6. Real-time PCR

Total RNA was extracted from adrenal medulla of hypertensive and normotensive rats using Trizol™ (Life Technologies, Inc.). Samples (1 μg) were further treated with Amplification Grade DNase I (Sigma-Aldrich). Reverse transcription for cDNA synthesis was carried out with a thermal cycler (Life Technologies, Inc.) using the SuperScript III First-Strand synthesis system according to manufacturer's protocol (Life Technologies, Inc.). The transcription rates of selected mRNAs were measured by real time PCR (Livak and Schmittgen, 2001) using the ABI Step One Plus Instrument (Life Technologies, Inc.). The PCR was performed in 25 μl buffer containing 1 μl cDNA, SYBR Green Master Mix (Life Technologies, Inc.) and 5 pmol of sequence specific primers of purinergic receptors and normalised by comparison with GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) RNA transcription levels. Thermal cycling conditions consisted of a pre-incubation step for 2 min at 50 °C, then denaturation for 10 min at 95 °C followed by 35 cycles for denaturation for 15 s at 95 °C and annealing/extension for 1 min at 60 °C. The comparative 2-ΔΔCT method was used for the relative quantification of gene expression (Livak and Schmittgen, 2001).

The primer sequences used in this study were: (1) $P2X_1$-F 5′GAGAGTCGGGCCAGGACTTC 3′–R-5′GCGAATCCCAAACACCTTGA3′; (2) $P2X_2$-F-5′TCCCTCCCCCACCTAGTCAC 3′–R-5′CACCACCTGCTCAGTCAGAGC3′; (3) $P2X_3$-F-5′CTGCCTAACCTCACCGACAAG 3′–R-5′AATACCCAGAACGCCACCC3′; (4) $P2X_4$-F-5′CCCTTTGCCTGCCCAGATAT3′–R-5′CCGTACGCCTTGGTGAGTGT3′; (5) $P2X_5$-F-5′GGATGCCAATGTTGAGGTTGA3′–R-5′TCCTGACGAACCCTCTCCAGT3′; (6) $P2X_6$-F-5′CCCAGAGCATCCTTCTGTTCC3′–R-5′GGCACCAGCTCCAGATCTCA3′; (7) $P2X_7$-F-5′GGGAGGTGGTTCAGTGGGTAA3′–R-5′GGATGCTGTGATCCCAACAAA3′; (8) $P2Y_1$-F-5′AACCGTGATGTGACCACTGA3′–R-5′TTCAACTTGTCCGTTCCACA3′; (9) $P2Y_2$-F-5′TGCTGGGTCTGCTTTTTGCT3′–R-5′ATCGGAAGGAGTAATAGAGGGT3′; (10) $P2Y_4$-F-5′TCGATTTGCAAGCCTTCTCT3′–R-5′CCATAGGAGACCAGGGTGAT3′; (11) $P2Y_6$-F-5′TGCTGCTACCCCCAGTTTAC3′–R-5′TGGCATAGAAGAGGAAGCGT3′; (12) $P2Y_{12}$-F-5′CTGTTTTTTGCTGGGCTCATC3′–R-5′GCGGATCTGGAAGAAAATCCT3′; (13) $P2Y_{13}$-F-5′GGATGCAGGGCTTCAACAA3′–R-5′GCAGCTGTGTCATCCGAGTGT3′; (14) $P2Y_{14}$-F-5′GGTGGGTTTCGCCTCATGT3′–R-5′CCTCAGGTGACCGGCATCT3′.

2.7. Materials and solutions

The following materials were used: collagenase type I from Sigma-Aldrich (Madrid, Spain); Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin fraction V, foetal calf serum, and antibiotics were from Gibco (Madrid, Spain). Trizol™ and Syber Green were from Life Technologies (Grand Island, NY USA). Amplification Grade DNase I and Fura-2 AM were from Sigma-Aldrich. Agatoxin-IVA was from Peptide Institute (Sandhausen, Germany) and ω-conotoxin GVIA was from Bachem Feinchemikalien (Bubendorf, Switzerland). All other chemicals used were reagent grade from Merck and Panreac Química (Madrid, Spain). Agatoxin-IVA and ω-conotoxin GVIA were dissolved in distilled water and stored frozen in aliquots at 0.1 mM. Nifedipine (10 mM) was prepared in dimethyl sulphoxide (DMSO) and protected from light. Final concentrations of drugs were obtained by diluting the stock solution directly into the extracellular solution. At these dilutions, solvents had no effect on the parameters studied.

2.8. Statistical analysis

For the fluorescence assay of $[Ca^{2+}]$ transients we normalised data by (F-F0)/F0 × 100, where F0 is the mean of basal $[Ca^{2+}]$ levels. In the graphs on $[Ca^{2+}]_c$ measurements, 0 was considered as baseline and 100% as Rmax. We analysed the maximum and minimum Ca^{2+} changes during 10 min periods, considering the mean fluorescence as a function of time. The mean of fluorescence ratio was taken into account because these dyes are ratiometric (340 and 380 nm excitation wavelengths). Peak I_{Ba} amplitude (ordinate) was measured at 5-ms after the initiation of the depolarising pulses. Data are means ± S.E.M. Differences were considered significant at $P < 0.05$. Two-population (NWR and SHR) comparisons were made with Student's *t*-test.

3. Results

3.1. Characterisation of barium currents in chromaffin cells from normotensive and hypertensive rats

To record inward currents through VDCCs we used 10 mM Ba^{2+} as charge carrier for two reasons: (i) to elicit a larger current that allowed a greater window for the pharmacological dissection of current subcomponents and (ii) to prevent Ca^{2+}-dependent

inactivation of VDCCs that differs among different subtypes of VDCCs expressed by chromaffin cells (Hernández-Guijo et al., 2001). Furthermore, using 10 mM Ca^{2+} as charge carrier an early component and a late, sustained component of I_{Ca} have been recently described in SHR and Wistar Kyoto Rat (WKR) chromaffin cells; the transient component is not present in NWR chromaffin cells. However, when 10 mM Ba^{2+} is used instead of Ca^{2+}, I_{Ba} had a single non-inactivating component in NWR, WKY and SHR chromaffin cells (Segura-Chama et al., 2011). This, together with the fact that in our three previous studies NWRs were used as normotensive controls of SHR, moved us to choose NWRs as controls also in the present study. NWRs have been also used as controls for SHRs in many other studies (see for instance (Habeck et al., 1985; Igarashi et al., 1977; Kiprov and Dimitrov, 1977; Kiprov et al., 1977; Sakai et al., 1984; Takahashi et al., 2011; Wei et al., 1977a, b)).

Under voltage-clamp conditions, I_{Ba} were elicited by 50-ms depolarising pulses applied from a holding potential of -80 mV, in 10-mV steps up to +60 mV. Potassium currents were suppressed by cell dialysis with a Cs^{+}- and TEA-based intracellular solution. Sodium currents (I_{Na}) inactivated in 3 ms and therefore, they could easily be separated from the non-inactivating I_{Ba} in the current traces, as seen in the example current traces of Fig. 1, from a NWR cell (panel A) and a SHR cell (panel B). Along the 50 ms pulse, I_{Ba} inactivated very slowly (see example current traces in Figs. 1A and 2A). I_{Ba} activated at −40/−30 mV, peaked at +10 mV in NWR, and at 0 mV in SHR cells; the reversal potential was at +60 mV for NWR (Fig. 1A) and at +50 mV for SHR (Fig. 1B). No evidence of low-threshold T-type (α_{1H}, Cav 3.2) VDCC current was seen in the current–voltage (I–V) curves. I_{Ba} peak amplitude, measured at 5 ms after the beginning of the depolarising pulse when I_{Na} was fully inactivated, amounted to 420 ± 22 pA in 26 NWR cells, and to 312 ± 25 pA in 44 SHR cells, 25.7% smaller ($P < 0.001$; Fig. 1C).

3.2. *Pharmacological dissection of I_{Ba} subcomponents linked to L-, N- and P/Q-subtypes of VDCCs in chromaffin cells from normotensive and hypertensive rats*

In our early study (Gandia et al., 1995) to define the subcomponents of I_{Ba} linked to different VDCC subtypes in rat chromaffin cells, we recoursed to selective blockers such as the DHP furnipidine for L channels, ω-conotoxin GVIA for N channels (GVIA) and ω-agatoxin IVA (AGAIVA) for P/Q channels or ω-conotoxin MVIIC (MVIIC) for N and P/Q channels. We added the compounds in a cumulative fashion within each individual cell. With this strategy we had previously found that the whole-cell I_{Ba} in rat chromaffin cells was carried out 50% through L channels, 30% through N channels and 20% through P/Q channels (Gandia et al., 1995). We have followed here a similar strategy to dissect the subcomponents of I_{Ba} in chromaffin cells from NWRs and SHRs. We have used supramaximal concentrations of nifedipine (3 μM), GVIA (1 μM) and AGAIVA (3 μM), known to selectively block L, N and P/Q components of whole-cell inward currents through VDCCs of chromaffin cells from various animal species (Garcia et al., 2006).

Fig. 2A shows two families of I_{Ba} traces representative of an example SHR chromaffin cell (left) and a NWR chromaffin cell (right). Blockers were sequentially added and I_{Ba} was depressed in a step-wise manner upon addition of AGAIVA followed by GVIA and finally nifedipine (Fig. 2B). Around 5% of I_{Ba} remained unblocked in the presence of the three compounds; this small fraction of residual current was blocked by 100 μM cadmium (not shown). Upon toxin and nifedipine washout, only the nifedipine-sensitive component of I_{Ba} recovered; the GVIA- and AGAIVA-components were irreversibly blocked (Fig. 2C).

Using this protocol we corroborated the findings of our previous study to dissect I_{Ba} subcomponent carried by L, N, and PQ channels

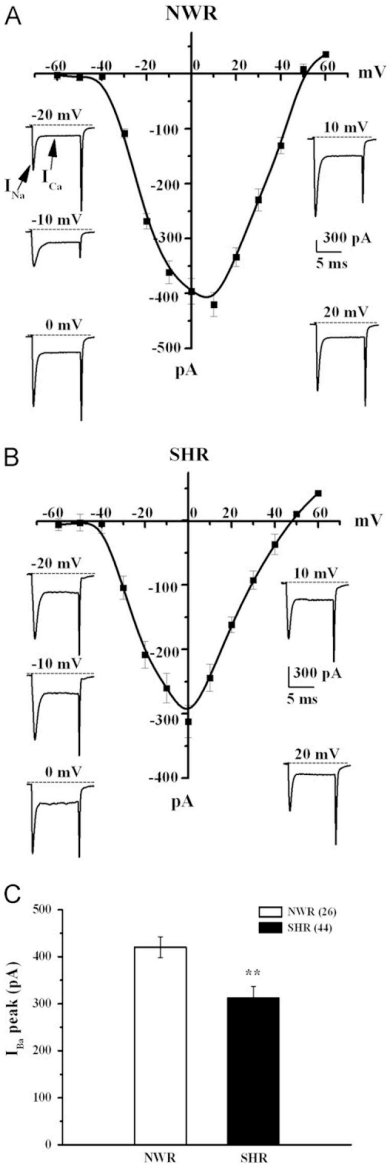

Fig. 1. Characteristics of barium currents (I_{Ba}) through VDCCs in chromaffin cells from normotensive (NWR, panel A) and hypertensive rats (SHR, panel B). Cells were voltage-clamped at −80 mV and 10-ms depolarising test pulses were applied in 10-mV steps at 10-s intervals, using 10 mM Ba^{2+} as charge carrier. Current (abscissa) versus voltage (ordinate) relationship in NWR (A) and SHR (B) cells are shown. Insets are original example current traces from a NWR chromaffin cell and a SHR chromaffin cells elicited by the test pulses indicated on top of each trace. Arrows indicate the fast-inactivating I_{Na} and non-inactivating I_{Ba} components. C, mean peak I_{Ba} (in pA, ordinate) calculated from the maximal current at 5-ms after initiation of the pulse, once I_{Na} was fully inactivated. Data are means ± S.E.M. of the number of cells shown in parentheses from at least eight different cultures.

(Gandia et al., 1995). However, from previous patch-clamp studies of chromaffin cells of other animal species, we know that the order of addition of the various toxins changes the fraction of current being

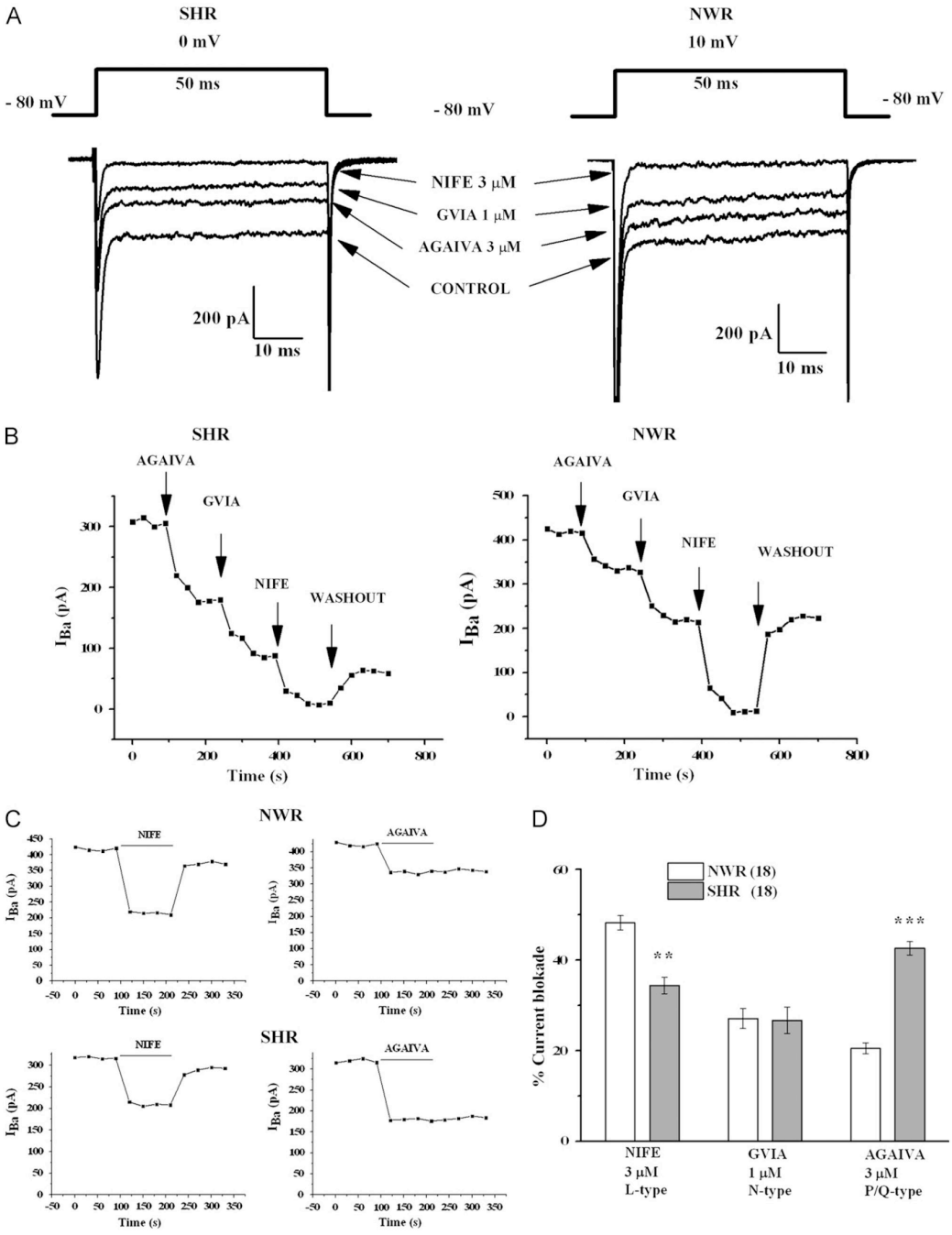

Fig. 2. Pharmacological dissection of I_{Ba} subcomponents linked to different VDCC subtypes, in chromaffin cells from normotensive (NWRs) and hypertensive rats (SHRs). Cells were voltage-clamped at −80 mV and I_{Ba} was generated by 50-ms depolarising pulses to +10 mV in NWR cells and to 0 mV in SHR cells, given at 30-s intervals. Cells were sequentially fast-perifused with extracellular solutions containing 10 mM Ba^{2+} in the absence (control current) and the presence of 3 μM ω−agatoxin IVA (AGAIVA), 1 μM ω-conotoxin GVIA (GVIA) and 3 μM nifedipine (NIFE); in these representative experiments, blockers were cumulatively added (panel B). A, example I_{Ba} traces from a SHR cell (left) and a NWR cell (right); each trace was selected from the time course of I_{Ba} traces, after 2 min of cell perifusion with each blocker, once the steady-state equilibrium of the new level of peak I_{Ba} was reached. *C*, time course of I_{Ba} blockade exerted by nifedipine (left) and AGAIVA (right) in different example NWR and SHR chromaffin cells. *D*, pooled averaged results on the extent of I_{Ba} inhibition (at equilibrium of peak I_{Ba}) elicited by each blocker (abscissa); note that only one blocker was tested in each cell. The % blockade was calculated by normalising to 100% the control I_{Ba} and expressing the I_{Ba} blockade elicited by each compound within each individual cell. Data are means ± S.E.M. of 18 cells from at least eight different cultures. $^{**}P < 0.01$, $^{***}P < 0.001$, with respect to NWR.

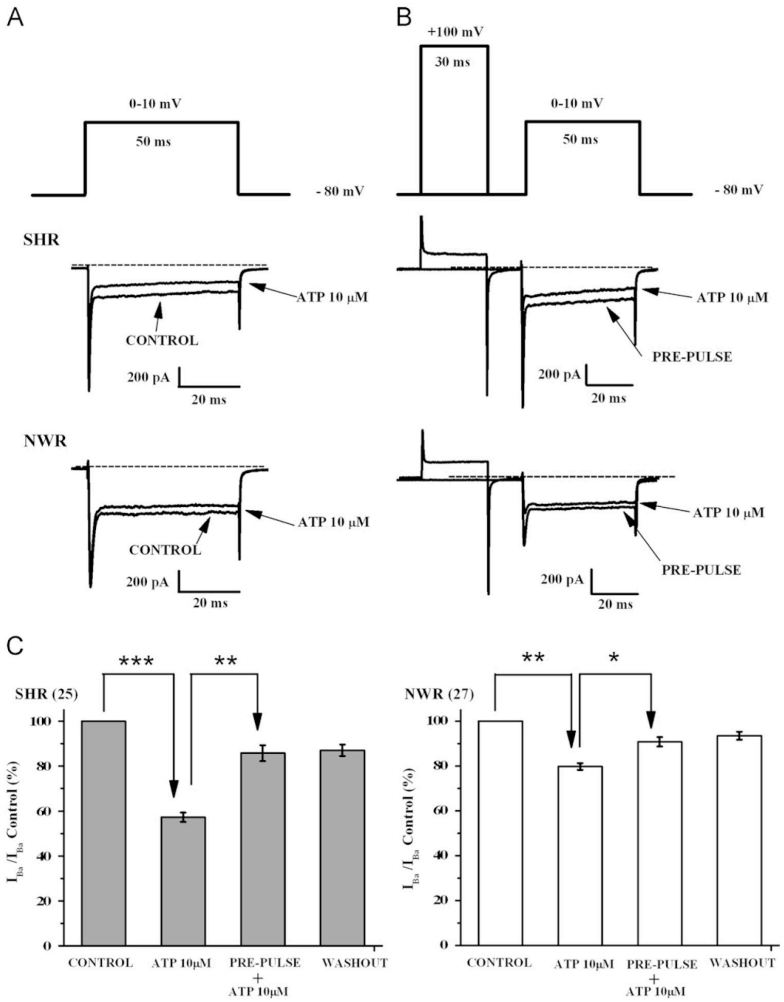

Fig. 3. Modulation by ATP of I_{Ba} in normotensive (NWR) and hypertensive (SHR) chromaffin cells A, whole-cell I_{Ba} were elicited by 50-ms depolarising test pulses to 0 mV (SHR cells) or +10 mV (NWR cells), applied at 30-s intervals from a holding potential of −80 mV. Example I_{Ba} traces obtained in the absence (control) and the presence of 10 μM ATP were obtained from a SHR (middle trace) and NWR cell (bottom trace) B, here a protocol similar to that of panel A was used, but a 20-ms pre-pulse to +100 mV was given 10 ms before the test pulse, in the presence of 10 μM ATP to remove the voltage-dependent G-protein mediated modulation of VDCCs. ATP was perifused for 30 s before the test pulse in both, panels A and B; C, averaged pooled results obtained in the number of cells shown in parentheses, from at least 8 different cultures. Data (ordinate) are normalised as % of control I_{Ba} (100% in the absence of ATP), within each individual cell. Data are means+S.E.M. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

blocked; thus here we decided to use a simple protocol in the pharmacological experiments aimed to determine the fraction of the whole-cell current carried by each VDCC subtype. To every cell, after entering the whole-cell configuration, test depolarising pulses to 0–10 mV were applied to elicit the maximal inward current. After stabilisation of the peak current, usually within the first 5 min, a given blocker was applied at the supramaximal concentrations mentioned above. Then, the fraction of current blocked in the cell being tested was calculated and normalised to calculate the fraction of current blocked. Examples of the time course of the blockade exerted by nifedipine and the toxins are displayed in Fig. 2C. Averaged pooled results from 18 cells, concerning the fraction of current inhibited by each blocker, are shown in Fig. 2D. While GVIA caused near 30% blockade in both cell types, significant differences were observed for nifedipine and AGAIVA. Thus, nifedipine caused 48.2 ± 1.6% I_{Ba} blockade in NWRs and 34.4 ± 1.8% in SHRs ($n=18$ cells for each NWRs and SHRs). The reverse was true for AGAIVA that elicited 21.5 ± 1.2% I_{Ba} blockade in NWRs and 42.6 ± 1.5% in SHRs ($n=18$ chromaffin cells from NWRs and SHRs). It seems therefore that the 30% reduction of I_{Ba} fraction carried by L channels was compensated by a doubling of I_{Ba} fraction carried by P/Q channels.

3.3. Modulation by ATP of I_{Ba} in chromaffin cells from normotensive and hypertensive rats

An autocrine–paracrine modulation by various neurotransmitters of VDCC currents has been amply studied in chromaffin cells (Garcia et al., 2006). Thus, it has been described that ATP slows

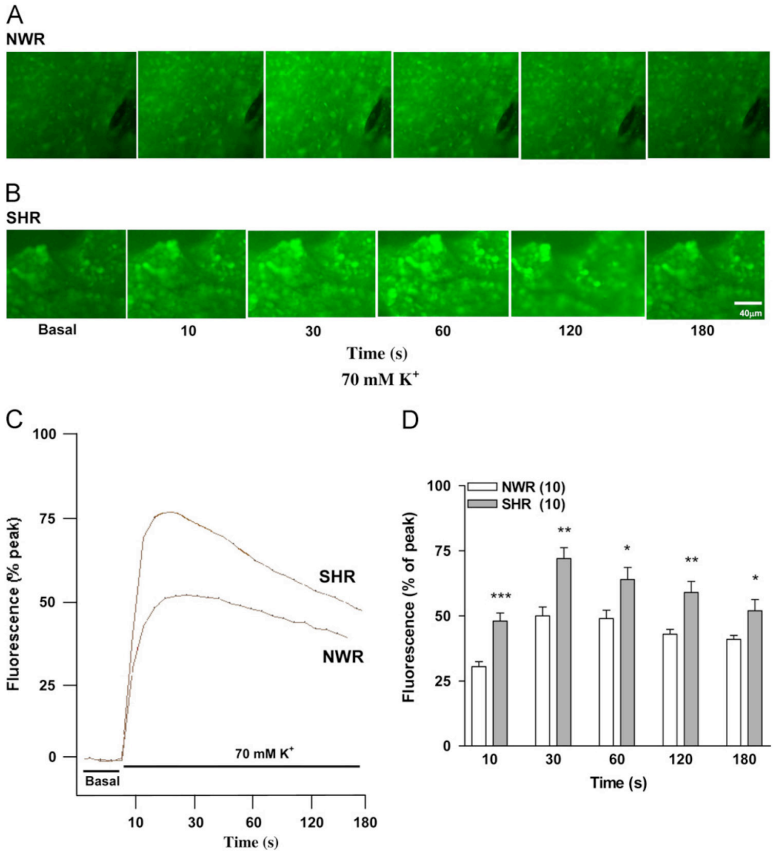

Fig. 4. Characteristics of the $[Ca^{2+}]_c$ elevations triggered by K^+ depolarisation of fura-2-loaded chromaffin cells in their intact environment of adrenal slices. Freshly prepared slices from a NWR (A) or SHR (B) loaded with fura-2. AM were continuously perifused with an oxygenated Tyrode solution containing 1.8 mM Ca^{2+}. After recording the fluorescence under resting conditions, slices were challenged with a high-K^+ solution (70 mM, with isosmolar reduction of Na^+) for 3 min. A, example sequential fluorescence images taken from a NWR adrenal slice (A) or SHR slice (B) under basal conditions and at the times indicated at the bottom bar. C, time course of the increase of fluorescence expressed in arbitrary fluorescence units (AFU, ordinate), corresponding to the images of panels A and B. D, pooled results from slices subjected to the protocol of panel A; maximum fluorescence reached at each time of the K^+ challenge was selected in each individual slice. Data are fluorescence means (ordinate) ± S.E.M. of 10 slices from at least 4 different NWRs or SHRs. $^*P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$ compared to NWR slices.

down and decreases the amplitude of I_{Ca} in voltage-clamped chromaffin cells (Gandia et al., 1993). Such modulation is predominantly exerted on non-L-type VDCCs and exhibits strong voltage-dependency; that is, the inhibition is removed by a positive strong depolarising prepulse (Garcia et al., 2006). In addition, it has been shown that the modulation of VDCCs by exogenous or endogenous ATP and opioids, via a G-protein membrane-delimited pathway, is altered in pathological human processes such as in pheochromocytoma, a chromaffin cell tumour characterised by a massive release of catecholamines into the circulation and the subsequent hypertensive crisis (Hernandez-Guijo et al., 2000).

We therefore considered of interest to study whether ATP could differently modulate I_{Ba} in chromaffin cells from NWRs and SHRs. I_{Ba} currents were elicited by 10-ms test depolarising pulses to 0 mV (SHR cells) or +10 mV (NWRs cells), from a holding potential of −80 mV, as indicated in the protocol of Fig. 3A (top). ATP (10 μM) nearly halved I_{Ba} amplitude in the example SHR cell of Fig. 3A (middle). The inhibition was smaller in the example NWR cell shown in Fig. 3A (bottom). Fig. 3B shows the pre-pulse protocol (top; used to remove the voltage-dependent modulation mediated by G-protein) and the example current traces elicited in the presence of ATP with or without the pre-pulse, in a SHR (middle trace) or NWR chromaffin cell (bottom trace). The short pre-pulse to +100 mV preceding the test pulse greatly removed the fraction of ATP-inhibited I_{Ba}.

Fig. 3C shows normalised pooled data on the effects of ATP on I_{Ba}, with or without pre-pulse application. In 27 NWR cells ATP caused 20 ± 1.5% I_{Ba} loss; the pre-pulse favoured the recovery of about half of the inhibited current. On the other hand, in 25 SHR cells ATP elicited 43 ± 2% of I_{Ba} loss; in its turn, the pre-pulse favoured the recovery of about 80% of the inhibited current. In other words, ATP had stronger modulatory effects of I_{Ba} in SHR, as compared with NWR chromaffin cells.

3.4. Characterisation of K^+-elicited $[Ca^{2+}]_c$ elevations in adrenal medullary slices of normotensive and hypertensive rats

During membrane depolarisation, Ca^{2+} enters the chromaffin cell to trigger the release of catecholamine (Douglas and Poisner,

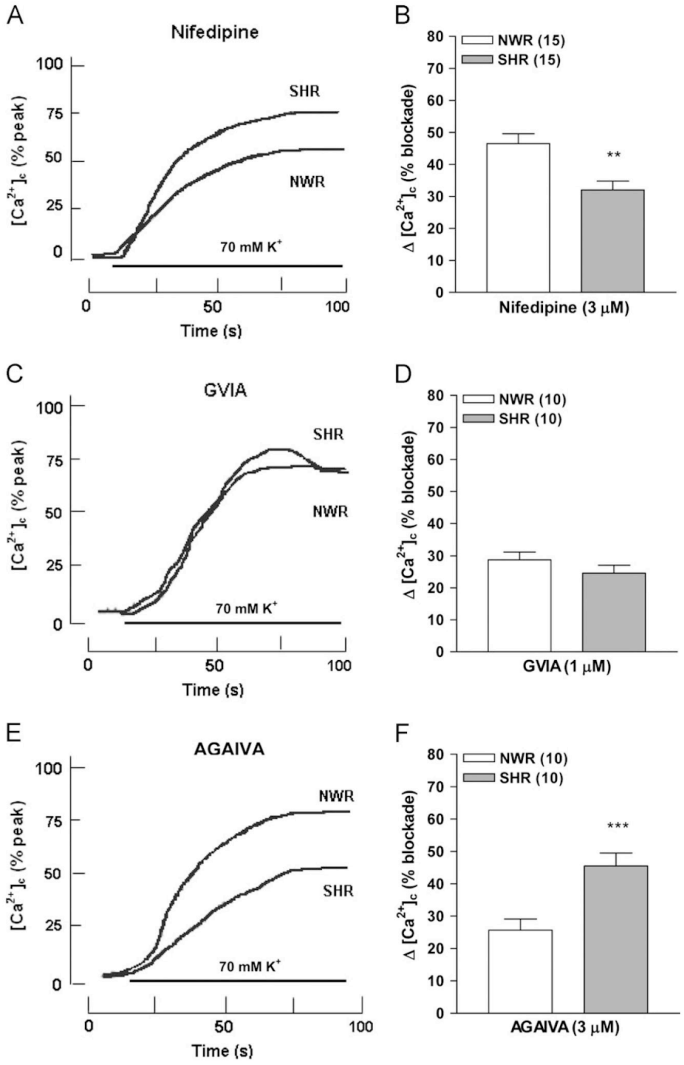

Fig. 5. Effects of VDCC blockers on the $[Ca^{2+}]_c$ elevation elicited by K^+ in fura-2-loaded adrenal slices of NWRs and SHRs. Data were normalised as % of the maximum fluorescence (*R* 340/380 nm) in each individual slice. Each slice was challenged with 70 mM K^+ and after 5-min resting period, it was incubated (during 5 min) either with 3 μM nifedipine, 1 μM ω-conotoxin GVIA (GVIA) or 3 μM ω-agatoxin IVA (AGAIVA). Then, in the presence of each blocker slices were challenged again with 70 mM K^+. The $[Ca^{2+}]_c$ elevations produced by 70 mM K^+ during blockade of L, N and P/Q-type inhibitors were normalised as % of maximum fluorescence obtained in the absence of each blocker. Panels A, C and E represent the time courses of the $[Ca^{2+}]_c$ elevations triggered by 90-s exposure to a high K^+ solution (bottom bars). Panels B, D and F represent normalised pool data obtained from experiments performed with the protocols of Panels A, C and E. Data are means ± S.E.M. of the % blockade of the K^+ elicited $[Ca^{2+}]_c$ increases, exerted by nifedipine (B), GVIA (D) and AGAIVA (F), in the number of NWR and SHR slices given in parentheses, from at least four different NWRs or SHRs. $^{**}P < 0.01$, $^{***}P < 0.001$ with respect to NWRs.

1961; Douglas and Rubin, 1961b). Since we found notable changes in the functional expression of VDCC subtypes, and in their regulation (Figs. 1–3) it was plausible that Ca^{2+} entry through each channel type could lead to different changes of $[Ca^{2+}]_c$ in chromaffin cells from NWRs and SHRs. We explored this possibility in freshly prepared adrenal medullary slices loaded with fura-2, a technique that we have recently optimised in the rat adrenal slice (Miranda-Ferreira et al., 2010).

Adrenal slices were challenged with a solution containing 70 mM K^+ (70 K^+; with isosmotic reduction of Na^+); this K^+ concentration causes chromaffin cell depolarisation to near 0 mV (Orozco et al., 2006), a voltage where peak I_{Ba} current was reached (Fig. 1) suggesting that all VDCC subtypes were being opened under these conditions. This allows the maximal Ca^{2+} entry and the gradual increase of the $[Ca^{2+}]_c$, as illustrated in the example sequence of fluorescence micrographs of Fig. 4A, taken

from a fura-2 loaded adrenal slice from a NWR. Fig. 4B shows a sequence of fluorescence micrographs taken from a SHR adrenal slice challenged with 70 K^+ (at the different times indicated on the bottom, after the K^+ challenge). The time course of fluorescence variations indicating $[Ca^{2+}]_c$ changes are shown in Fig. 4C. The time course of such $[Ca^{2+}]_c$ signal follows a rapid initial rising phase, and a later slow decay phase, as shown in Fig. 4C, constructed with data taken from the images of Fig. 4A,B. Pooled averaged results from 10 experiments are shown in Fig. 4D; at all times analysed the K^+-elicited augmentation of fluorescence was significantly higher in SHR, compared with NWR slices.

3.5. The effects of VDDC blockers on the elevations of $[Ca^{2+}]_c$ produced by K^+ in adrenal slices from normotensive and hypertensive rats

To find out the contribution of each VDCC subtype to the K^+-elicited $[Ca^{2+}]_c$ elevation, we recoursed to the selective blockers and at the concentration used to dissect I_{Ba} subcomponents (Fig. 2). Each individual slice was challenged with a 70 K^+ solution to test the initial $[Ca^{2+}]_c$ elevation. After 5 min, it was incubated with either 3 μM nifedipine, 1 μM GVIA or 3 μM AGAIVA for an additional 5-min period. Then a second K^+ challenge was applied still in the presence of the blocker. After 5 min of blocker washout, a third K^+ challenge was applied to test for the viability of the preparation and response recovery.

Example records of the time courses of the normalised $[Ca^{2+}]_c$ elevations produced by a 90-s time period of slice exposure to 70 K^+ in the presence of 3 μM nifedipine are shown in Fig. 5A. There is a gradual $[Ca^{2+}]_c$ increase above basal that reached a plateau at 1 min after K^+ exposure; the rising phase of $[Ca^{2+}]_c$ was faster in SHR slices. This difference could be due to the fact that the blockade by nifedipine of the $[Ca^{2+}]_c$ increase was higher in NWR slices (46.5 ± 3.1%) than in SHR slices (32 ± 2.8%) (Fig. 5B).

The example $[Ca^{2+}]_c$ traces taken in the presence of 1 μM GVIA overlapped (Fig. 5C), indicating that the blockade of the response to K^+ was similar in NWR and SHR slices, as shown in the bar graph of Fig. 5D: GVIA caused an inhibition of the $[Ca^{2+}]_c$ response of 28.8 ± 2.4% in NWR slices and of 24.6 ± 2.5% in SHR slices. At 3 μM, AGAIVA elicited a greater reduction of the $[Ca^{2+}]_c$ signal in SHR slices, compared with NWR slices; this could be the reason for the substantial slower rate of rise of $[Ca^{2+}]_c$ in the former, as compared with the latter (Fig. 5E). This is consistent with the higher blockade by AGAIVA of the K^+-evoked $[Ca^{2+}]_c$ elevation in SHR slices (45.5 ± 4%), compared with that produced in NWR slices (25.7 ± 2.8%) (Fig. 5F).

3.6. Relative expression of different purinergic receptors in the adrenal medulla of normotensive and hypertensive rats

Different modulation by ATP of I_{Ba} in voltage-clamped NWR and SHR cells could be due to the differential relative expression of purinergic receptor subtypes that are known to be expressed by adrenal medullary chromaffin cells (Burnstock, 2006, 2007). We found that this was clearly the case, as demonstrated by the experiments shown in Fig. 6.

Particularly striking was the enhanced expression of the P_{2X1} receptor mRNA in SHRs (11.6 ± 1.6 AU) compared with NWRs (1 ± 0.6 AU). P_{2Y2} receptors were also augmented in SHRs (12.5 ± 2.5 AU) compared with NWRs (1 ± 0.2 AU). P_{2X3} and P_{2X6} receptors were also augmented in SHRs by around 2- and 3-fold respectively, compared with NWRs. Some other purinergic receptor subtypes were expressed more in NWRs, compared with SHRs. This was the case of P_{2X2}, P_{2X7}, P_{2Y1}, P_{2Y12}, P_{2Y13} and P_{2Y14}.

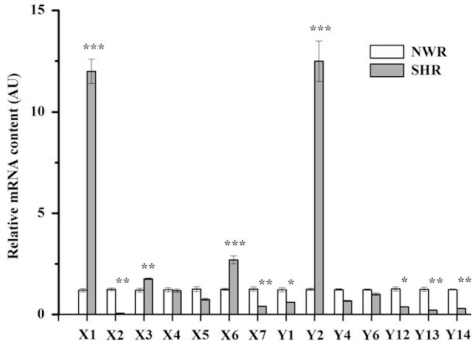

Fig. 6. Relative mRNA expression coding for the different subtypes of purinergic receptors in adrenal medullary tissues of NWRs and SHRs. mRNA was measured by real time PCR (see Methods). Data are expressed as relative arbitrary units (AU, ordinate); they are means ± S.E.M. of 20 adrenals each from NWRs and SHRs. $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0005$ with respect to NWRs.

4. Discussion

The central finding of this study is the diminution of L VDCC density and the concomitant augmentation of PQ channel density in adrenal chromaffin cells from hypertensive, compared with normotensive rats. These changes may be relevant in the context of the following drastic differences in the kinetics and regulation of L and PQ channels known to occur in chromaffin cells: (1) Ca^{2+} channel currents are modulated by neurotransmitters (including ATP) in a voltage-dependent (PQ channels) or voltage-independent manner (L channels) (Albillos et al., 1996a; Gandia et al., 1993; Gandia et al., 1998; Hernandez-Guijo et al., 1999); (2) depending on the experimental condition, L channels may be preferentially coupled to exocytosis (Lopez et al., 1994) or PQ channels may dominate the control of catecholamine release (Lara et al., 1998); (3) cell stimulation with action potentials produce a secretory response mostly controlled by PQ channels, while secretion stimulated with depolarising pulses is controlled by other channel subtypes (Chan et al., 2005); (4) L channels are tightly coupled to endocytosis while PQ channels are more weakly coupled to endocytosis (Perissinotti et al., 2008; Rosa et al., 2007, 2011); (5) L channels are more resistant to voltage-dependent inactivation while PQ channels are more prone to such inactivation (Hernandez-Guijo et al., 1997; Villarroya et al., 1999); and (6) L channels undergo Ca^{2+} dependent inhibition at a rate slower than PQ channels (Hernández-Guijo et al., 2001).

The relative density of L channels decreased by 30% and PQ channels increased by 100% in SHR compared with NWR chromaffin cells (Fig. 2). These changes could be interpreted in the context of previous experiments done in voltage-clamped chromaffin cells in which we provided evidence to support the presence of a crosstalk between L- and non-L-type of VDCCs: during cell activation, the Ca^{2+} entering the cell through the lesser inactivating L channels serves to favour the Ca^{2+}-dependent inactivation of PQ channels (Rosa et al., 2009). In this context, it is plausible that the lower density of L channels in SHR chromaffin cells could lead to lesser Ca^{2+} entry through this pathway, thereby decreasing the rate of PQ channel inactivation. This, together with the fact that PQ channel density rose, could explain the higher $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ found in adrenal slices of SHR, with respect to NWRs. It could also explain that the $[Ca^{2+}]_c$ elevations triggered by Acetylcholine or K^+ were also higher in SHR, with respect to NWR chromaffin cells. However, the greater $[Ca^{2+}]_c$ elevations elicited by challenging SHR cells with CRT or

FCCP (Miranda-Ferreira et al., 2010) cannot simply be explained by changes in VDCC densities. Impairment of Ca^{2+} clearance mechanisms, in addition to changes in VDCC densities, may therefore contribute to the considerably greater $[Ca^{2+}]_c$ signals occurring in SHR, compared with NWR chromaffin cells upon their stimulation with Acetylcholine or K^+ in our previous study (Miranda-Ferreira et al., 2010) and with K^+ in the present study.

Another interesting finding of this study was the greater modulation by ATP of I_{Ba} in SHR, compared with NWR chromaffin cells. It is known that in these cells ATP exerts a regulatory role through both inhibition and augmentation of Ca^{2+} current via a pertussis toxin (PTX)-sensitive pathway (Diverse-Pierluissi et al., 1991). Furthermore, in bovine chromaffin cells we reported that ATP rapidly and reversibly inhibits calcium channel currents also via activation of a PTX-sensitive regulatory *G* protein and current-facilitating conditioning depolarising prepulses leading to reversal of ATP-elicited current inhibition (Gandia et al., 1993). In the present study, in rat chromaffin cells we have also found that ATP reversibly inhibited I_{Ba} and that facilitating depolarising prepulses reversed the ATP-inhibited current. However, this ATP regulation was more pronounced in SHR, as compared with NWR chromaffin cells (Fig. 3).

The greater efficacy of ATP and facilitating depolarising prepulses to regulate the VDCCs of SHR compared with NWR chromaffin cells could be explained by the tenfold augmentation of mRNA expression for P_{2Y2} purinergic receptors in the former, compared with the latter (Fig. 6). This drastic augmented expression of P_{2Y2} receptors that are known to be involved in the G protein-mediated VDCC current inhibition in bovine chromaffin cells (Gandia et al., 1993) could result from a feedback rebound regulatory mechanism in an attempt of SHR chromaffin cells, to limit Ca^{2+} entry and excess catecholamine release that could be a salient pathogenic factor in the development of a hypertensive state. We also found over tenfold higher mRNA expression coding for P_{2X1} receptors (Fig. 6); because they are Ca^{2+} permeable channels, enhanced function of P_{2X1} receptors could be an additional factor to enhanced Ca^{2+} signalling in SHR cells.

These findings are interesting in the context of the observation that a physiological modulatory role for endogenously released ATP has been suggested to occur in chromaffin cells. In these cells ATP is co-stored (Winkler and Westhead, 1980) and co-released with catecholamines (Castillo et al., 1992; Rubin and Jaanus, 1967; Stevens et al., 1975). That the endogenously released ATP causes the inhibition of calcium channel currents in chromaffin cells was demonstrated by indirect flow-stop experiments (Albillos et al., 1996b; Currie and Fox, 1996; Doupnik and Pun, 1994) and by more direct approaches showing that a chromaffin vesicle lysate containing endogenous ATP causes a modulation of VDCC current similar to that exerted by exogenously applied ATP (Albillos et al., 1996b).

How the present findings could fit in the enhanced catcholamine release response reported to occur in adrenal chromaffin cells from hypertensive versus normotensive rats, deserves a last comment. The following observations are compatible with greater quantal release responses in SHR chromaffin cells (Miranda-Ferreira et al., 2009, 2008): (1) PQ channels are more coupled to exocytosis (Chan et al., 2005; Lara et al., 1998) and thus, enhanced expression of these channels in SHR cells could contribute to provide more Ca^{2+} to strategically located exocytotic sites; (2) L channels are more tightly coupled to endocytotic than exocytotic sites (Perissinotti et al., 2008; Rosa et al., 2007, 2011) and thus, decreased expression of these channels in SHR cells could lead to decreased endocytosis and to a greater exocytotic response; (3) decreased Ca^{2+} entry through L channels may impair the Ca^{2+}-dependent inhibition of PQ channels (Rosa et al., 2009) and thus, Ca^{2+} entering through these uninhibited channels, which have higher density in SHR chromaffin cells, could enhance the secretory response. In this context, the greater ATP regulation of VDCCs should theoretically lead to lesser Ca^{2+} entry and smaller secretory responses in SHR cells. Also the smaller (25%) whole-cell current through VDCCs we found in SHR cells (Fig. 1C) should lead to smaller secretory responses. Because secretion of SHR cells to different secretagogues was markedly enhanced with respect to NWR chromaffin cells (Miranda-Ferreira et al., 2009, 2008), we must conclude that in addition to Ca^{2+} entry through VDCCs (an element of the functional triad) the redistribution into, and the subsequent release of Ca^{2+} from the ER and mitochondria (the other two elements of the functional triad) may also be altered and contribute to enhanced catecholamine release responses (Miranda-Ferreira et al., 2009).

In conclusion, the following findings emerge from this comparative study on SHR, compared with NWRs: (1) SHR chromaffin cells have a lower density of L-type channels that is compensated by a higher density of PQ channels; (2) more visible current modulation by ATP that could be linked to a 10-fold higher mRNA levels of purinergic receptors of the P_{2Y2} subtype in SHR cells; and (3) a higher contribution of PQ channels to the $[Ca^{2+}]_c$ transients generated by K^+ in SHR, with respect to NWR chromaffin cells. The results of the present study shed additional light on the relative contribution of the three elements of the functional triad that were formed by the VDCCs, the ER, and mitochondria (Garcia et al., 2006, 2012) to the greater and longer duration catecholamine release responses elicited by different secretagogues in chromaffin cells of hypertensive, compared with normotensive rats. These results may also inspire novel approaches to treatment of hypertension in the clinic, by addressing novel pharmacotherapeutic targets.

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DISCUSIÓN

5.-DISCUSIÓN

Las ratas de la cepa SHR (de "*Spontaneously Hypertensive Rat*") utilizadas en la presente Tesis Doctoral constituyen un modelo experimental ampliamente utilizado en diversos estudios orientados al esclarecimiento de los mecanismos fisiopatológicos implicados en el desarrollo de la PA esencial (Okamoto y Aoki, 1963; Pinto y col., 1998).

Entre estos posibles mecanismos, se postula que una alteración de la actividad del eje simpatoadrenal, con un incremento de la liberación de catecolaminas, puede ser una de las bases fisiopatológicas implicadas en la hipertensión arterial (HTA). De hecho, se ha observado que los niveles plasmáticos de adrenalina y noradrenalina se encuentran elevados en ratas SHR (Iriuchijima, 1973; Grobecker y col., 1975; Pak, 1981), de forma similar a lo que ocurre en los pacientes que sufren HTA esencial (Goldstein, 1983). En estudios previos realizados en glándulas adrenales intactas de ratas SHR se ha observado que la liberación de catecolaminas inducida por la aplicación del neurotransmisor fisiológico acetilcolina (ACh) o por concentraciones elevadas de K^+, es mayor en ratas SHR, en comparación con ratas normotensas control (Donohue y col., 1988; Tsuda y Masuyama, 1991; Lim y col., 2002; Miranda-Ferreira y col., 2008; Miranda-Ferreira y col., 2009).

En la presente Tesis Doctoral hemos caracterizado la liberación de catecolaminas adrenomedulares a nivel de célula cromafín única utilizando un estímulo fisiológico como la ACh frente a un estímulo despolarizante por alto K^+ comparando la secreción obtenida en células de ratas control con la obtenida en célula de ratas hipertensas (SHR). Hemos utilizado para ello la técnica de registro amperométrico de la secreción de catecolaminas, que nos permite una alta resolución temporal (en un rango de milisegundos a segundos) pudiendo registrar eventos secretores unitarios, correspondientes a la liberación de una sola vesícula de secreción. Esto nos ha permitido caracterizar la cinética de la liberación de catecolaminas en estas condiciones experimentales en los dos modelos celulares estudiados.

En este estudio hemos encontrado que la respuesta exocitótica cuántica de liberación de catecolaminas tras la aplicación de estímulos cortos (2 s) de ACh o K^+, era de mayor magnitud (un incremento de 3 veces mayor de liberación de contenido de las vesículas), más duradero en el tiempo tras la aplicación del estímulo, espigas más altas

con una cinética de liberación mucho más rápida en la rata SHR que en su congénere normotenso (WKY) (Miranda-Ferreira y col., 2008). Esta original observación podría explicar por qué la respuesta al estrés de los pacientes hipertensos produce una mayor agresión catecolaminérgica sobre el corazón y vasos; esto podría ser una de las causas de mayor riesgo cardiovascular que poseen los pacientes hipertensos pues las catecolaminas aumentan el gasto cardíaco pero a expensas de un sustancial aumento del consumo de oxígeno por el corazón.

Ante estos resultados, nos planteamos la hipótesis de que estas drásticas diferencias en la secreción de catecolaminas entre las células cromafines de ratas control y las de ratas SHR pudieran estar relacionadas con diferencias en la homeostasia del Ca^{2+} entre los dos modelos celulares. A este respecto, como hemos descrito previamente, nuestro grupo ha postulado la hipótesis de que existiría una unidad funcional compuesta por los CCDV, el RE y la mitocondria que estaría regulando los microdominios de Ca^{2+} necesarios para regular las etapas tempranas y tardías del proceso exocitótico y que hemos denominado la "Triada funcional" (Figura 9) (Garcia y col., 2006). Por ello, nos planteamos caracterizar la posible implicación de los elementos que integran esta triada funcional (esto es, los CCDV, el RE y la mitocondria), en la regulación de la secreción de catecolaminas en animales control e hipertensos, utilizando para ello tanto células cromafines aisladas como un modelo más fisiológico basado en el uso de rodajas de glándulas adrenales de ratas control y ratas SHR.

En los experimentos realizados en células cromafines de rata aisladas, utilizando la técnica de registro amperométrico de eventos secretores únicos y diversos fármacos para manipular los niveles de Ca^{2+} en el RE y la mitocondria, hemos encontrado que la liberación de Ca^{2+} del RE (con la mezcla CRT) o desde la mitocondria (con el protonóforo FCCP), produce respuestas secretoras mayores en células SHR, en comparación con las controles, de forma similar a lo que ocurre tras la estimulación de la secreción con ACh o K^+. Hemos observado también que el pretratamiento de las células cromafines con CRT y/o FCCP produce una mayor secreción en respuesta a la estimulación con ACh o K^+, en comparación con células no tratadas, siendo esta potenciación similar en células controles y en células de rata SHR (Miranda-Ferreira y col., 2009).

Quisimos ver, de una forma más fisiológica, la gestión del Ca^{2+} por el RE y la mitocondria, utilizando para ello rodajas de médula adrenal en las que, utilizando sondas fluorescentes con diferentes afinidades, pudimos medir los niveles de Ca^{2+} libre a nivel del citosol, del RE y de la mitocondria, respectivamente. En estos experimentos hemos observado que las rodajas de las ratas SHR tienen incrementadas las $[Ca^{2+}]$ basales a nivel del citosol y de la mitocondria, en comparación con las rodajas de ratas controles. Además, se alcanzan mayores $[Ca^{2+}]_c$ en células cromafines de ratas SHR tras la estimulación con ACh o K^+. También se apreciaron mayores $[Ca^{2+}]_c$ en rodajas de glándula adrenal de las ratas SHR tras la movilización de Ca^{2+} desde el RE o desde la mitocondria (Miranda-Ferreira y col., 2010). Los resultados obtenidos en estos experimentos realizados en rodajas también nos mostraron que se produce una mayor captación de Ca^{2+} en el RE cuando se favorece la movilización del Ca^{2+} de la mitocondria con el uso del protonóforo FCCP, lo que supondría una mayor capacidad de almacenamiento de Ca^{2+} del RE en las células cromafines de las ratas SHR, produciéndose consecuentemente una mayor liberación de Ca^{2+} desde el RE al aplicar la mezcla de vaciado CRT (Miranda-Ferreira y col., 2010).

Estos datos sugieren que las alteraciones de la capacidad de la mitocondria para captar y liberar el Ca^{2+}, en el denominado "ciclo del calcio mitocondrial" (Duchen, 2000; Garcia y col., 2012; Garcia-Sancho y col., 2012) podrían estar implicadas en la patogénesis de la HTA. De hecho, otros autores han observado un incremento de la captación de Ca^{2+} en la mitocondria a través del uniportador de Ca^{2+} (Arab y col., 1990) en células de músculo liso durante la HTA, lo que podría ocasionar un incremento de la $[Ca^{2+}]_M$ como el observado en nuestro estudio.

Puesto que las mayores $[Ca^{2+}]_c$ que se alcanzan en células cromafines de ratas SHR en respuesta a la despolarización producida por la estimulación con ACh o K^+, podrían correlacionarse con una mayor expresión de un determinado subtipo de CCDV en ratas SHR, nos planteamos realizar un estudio electrofisiológico orientado a la caracterización funcional de los distintos subtipos de CCDV en estas células, utilizando una combinación de técnicas de registro electrofisiológico con potentes bloqueantes de los diferentes subtipos de CCDV. Los resultados obtenidos en este estudio nos mostraron que en las células cromafines de ratas SHR hay una menor densidad de CCDV del subtipo L (48,2% *vs* 34,4% en NWR y SHR, respectivamente) que se vio compensada con un aumento en la densidad de los CCDV del subtipo PQ (21,5% vs

42,6%, en NWR y SHR, respectivamente). No se apreciaron diferencias significativas en la densidad de CCDV del subtipo N (de Pascual y col., 2013).

Puesto que los CCDV del subtipo PQ son más susceptibles a ser modulados por ATP (Gandia y col., 1993b; Garcia y col., 2006) quisimos estudiar los efectos de la aplicación de este nucleótido sobre las corrientes de entrada a través de los CCDV en células cromafines de ratas controles y SHR. Observamos curiosamente una mayor efecto modulador de la I_{Ba} por ATP en células SHR, en comparación con las células de ratas normotensas. Esta mayor modulación podría corresponderse con la mayor presencia (de hasta unas 10 veces) de receptores purinérgicos del subtipo P_{2Y2} que hemos observado, mediante estudios de PCR cuantitativa, en las células de ratas SHR, en comparación con el control (de Pascual y col., 2013). Este drástico incremento de la expresión de receptores P2Y2, que están implicados en la modulación de los CCDV del subtipo PQ mediada por proteínas G (Gandia y col., 1993b), podría ser el resultado de un mecanismo adaptativo de las células cromafines de ratas SHR para limitar la entrada de Ca^{2+} y el exceso de liberación de catecolaminas (de Pascual y col., 2013).

Finalmente, cabe destacar que también hemos observado un marcado incremento en el ARNm para los receptores purinérgicos del subtipo P_{2X1} en células SHR, en comparación con las células control; puesto que se trata de un receptor de tipo ionotrópicos permeable a Ca^{2+}, esta mayor expresión podría contribuir también a generar una mayor señal citosólica de Ca^{2+} y, por ende, una mayor respuesta secretora de catecolaminas en células SHR (de Pascual y col., 2013).

Los resultados resaltan el papel de la secreción aumentada de catecolaminas (particularmente de adrenalina) en la génesis de un estado hipertensivo arterial. Esa mayor respuesta se debe por una parte a los niveles más elevados del calcio en SHRs, tanto en el citosol como en la mitocondria, y a su mayor movilización durante la estimulación de la célula cromafín.

CONCLUSIONES

6.-CONCLUSIONES

Los resultados obtenidos durante el desarrollo experimental de la presente Tesis Doctoral nos permiten concluir que:

1) La respuesta exocitótica cuántica de liberación de catecolaminas tras estímulos cortos de ACh o K^+ es de mayor magnitud, más duradera, y con una cinética de liberación más rápida en la rata SHR hipertensa que en su congénere normotenso (WKY).

2) La depleción del depósito de calcio del RE o la disipación del gradiente mitocondrial de protones, produce efectos similares sobre la respuesta secretora en ambos tipos de células cromafines, por lo que las notables diferencias en la cinética de la exocitosis no parecen relacionarse con una perturbación de los movimientos intracelulares del calcio.

3) Sin embargo, en rodajas de glándula adrenal, las ratas SHR tienen incrementado el calcio citosólico y mitocondrial con una cinética de activación de la entrada de calcio más elevada y de aclaramiento más lenta que en las ratas normotensas. La sobrecarga de Ca^{2+} citosólico hace que también se incremente el Ca^{2+} mitocondrial, afectándose así el ciclo intracelular del calcio, lo que puede producir una disfunción en la bioenergética mitocondrial.

4) En las células de rata SHR hay una baja densidad de los CCDV del subtipo L que parece estar compensada con un aumento en la densidad de los CCDV del subtipo PQ, no habiendo diferencias significativas en la densidad de CCDV del subtipo N.

5) Las células de ratas SHR muestran unos niveles mayores de ARNm de receptores purinérgicos del subtipo P2Y2 implicados en la modulación por ATP de los CCDV.

Como conclusión general cabe resaltar que los resultados de esta tésis consolidan la idea de la destacada contribución de la médula adrenal a una mayor secreción de catecolaminas tras la estimulación colinérgica de las células cromafines, como responsable del desarrollo de la hipertensión arterial. Ello se debe a una mayor movilización del calcio mitocondrial y a un mayor contenido cuántico de catecolaminas en las vesículas cromafines de la rata SHR, en comparación con la normotensa. Estos datos podrían generar ideas nuevas para el desarrollo de nuevos fármacos antihipertensivos cardioprotectores

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7.-BIBLIOGRAFIA

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